

The Zur of *Xanthomonas campestris* functions as a repressor and an activator of putative zinc homeostasis genes via recognizing two distinct sequences within its target promoters

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ABSTRACT

It has been long considered that zinc homeostasis in bacteria is maintained by export systems and uptake systems, which are separately controlled by their own regulators and the uptake systems are negatively regulated by Zur which binds to an about 30-bp AT-rich sequence known as Zur-box present in its target promoters to block the entry of RNA polymerase. Here, we demonstrated *in vivo* and *in vitro* that in addition to act as a repressor of putative Zn²⁺-uptake systems, the Zur of the bacterial phytopathogen *Xanthomonas campestris* pathovar *campestris* (*Xcc*) acts as an activator of a Zn²⁺ efflux pump. The *Xcc* Zur binds to a similar Zur-box with ~30-bp AT-rich sequence in the promoters of the genes encoding putative Zn²⁺-uptake systems but a 59-bp GC-rich sequence with a 20-bp inverted repeat overlapping the promoter's -35 to -10 sequence of the gene encoding a Zn²⁺-export system. Mutagenesis of the inverted repeat sequence resulted in abolishment of the *in vitro* binding and the *in vivo* and *in vitro* activation of the export gene's promoter by Zur. These results reveal that the *Xcc* Zur functions as a repressor and an activator of putative zinc homeostasis genes via recognizing two distinct sequences within its target promoters.

INTRODUCTION

Like many other transition metal ions, zinc has paradoxical biological effects. On one hand, zinc is an essential trace element for almost all living organisms and works as a catalytic cofactor for various enzymes and a structural component in numerous proteins, on the other hand, when its intracellular concentration exceeds a threshold, it forms unspecific complex compounds in the cell and then causes toxic effects to the organism (1,2). In consequence, the intracellular Zn²⁺ level must be precisely regulated. In bacteria, the balance between Zn²⁺ import and export, known as zinc homeostasis, is maintained mainly through the coordinated expression of import systems and export systems which are separately regulated by their own regulators (3). Many bacteria use the zinc uptake regulator Zur, a Zn²⁺-sensing metalloregulatory protein belonging to the Fur (Ferric uptake regulator) regulatory protein family, to repress the transcription of Zn²⁺-uptake systems to maintain a delicate homeostasis (3,4). Zur was first described as a negative transcriptional regulator of genes encoding Zn²⁺-uptake systems in *Escherichia coli* (5) and *Bacillus subtilis* (6). Subsequently, it has been characterized in a number of other bacterial species including *Listeria monocytogenes* (7), *Staphylococcus aureus* (8), *Salmonella serovar* (9), *Pasteurella multocida* (10), *Xanthomonas campestris* (11), *Mycobacterium tuberculosis* (12) and *Streptomyces coelicolor* (13). The major role of Zur in these bacteria is also to repress the transcription of genes encoding Zn²⁺-uptake

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

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systems to maintain zinc homeostasis. Recently, it has been demonstrated that the Zur of *M. tuberculosis* and *S. coelicolor* also controls intracellular Zn²⁺ mobilization through regulating some Zn²⁺-binding ribosomal proteins besides repressing Zn²⁺ uptake (12,13).

The molecular mechanism by which Zur represses the expression of Zn²⁺-uptake systems has been studied in some details. In *E. coli*, the Zur protein binds to an about 30-bp AT-rich sequence known as Zur-box overlapping the -35 to -10 region of the promoter of the Zn²⁺ uptake operon *znuABC* to block the entry of the RNA polymerase and thus suppresses the transcription of *znuABC* (14,15). Similarly, the Zur proteins of *B. subtilis*, *M. tuberculosis* and *S. coelicolor* also bind to a ~30-bp AT-rich sequence overlapping the -35 to -10 region of their target promoters (12,16,17). The conservation in sequence and location of the Zur-binding sites of *E. coli*, *B. subtilis*, *M. tuberculosis* and *S. coelicolor* suggests that these Zur proteins may use a similar mechanism to repress the expression of the Zn²⁺-uptake systems.

To the best of our knowledge, there is not report about a Zur to be involved in the regulation of Zn²⁺-export systems. Our previous work showed that the *zur* mutant of the phytopathogenic bacterium *Xanthomonas campestris* pathovar *campestris* (*Xcc*), the causal agent of black rot disease of cruciferous crops (18), is significantly more sensitive to high zinc concentrations and accumulates significantly more zinc than the wild-type strain (11). In this

study, we demonstrated that the Zur protein of *Xcc* not only represses the expression of genes encoding putative Zn²⁺-uptake systems but also activates the expression of a gene encoding a Zn²⁺ efflux pump via directly binding to *cis*-acting elements overlapping the promoters' -35 to -10 region of these genes. Interestingly, the results displayed that the Zur-binding sequence in the genes encoding the putative Zn²⁺-uptake systems is distinct to that in the Zn²⁺-export gene, indicating that the *Xcc* Zur can recognize at least two distinct targets.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

The bacteria and plasmids used in this work are listed in Table 1. *Escherichia coli* strains were grown in LB medium (19) at 37°C. *Xcc* strains were grown in NYG medium (20) at 28°C. Antibiotics were used at the following final concentrations: rifampicin, 50 µg/ml; kanamycin, 25 µg/ml; ampicillin, 100 µg/ml; chloramphenicol, 100 µg/ml and tetracycline, 15 µg/ml for *E. coli* and 5 µg/ml for *Xcc*.

DNA manipulation

DNA manipulation was performed following the procedures described by Sambrook *et al.* (21). The conjugation between the *Xcc* and *E. coli* strains was performed as described by Turner *et al.* (22). Restriction enzymes and

Table 1. Bacterial strains and plasmids used in this work

Strains or plasmids	Relevant characteristics ^a	Reference or source
<i>E. coli</i>		
JM109	<i>RecA1, endA1, gyrA96, thi, supE44, relA1</i> Δ (<i>lac-proAB</i>)/F' [<i>traD36, lacI^f, lacZ</i> Δ M15]	(32)
BL21(DE3)pLysS	F ⁻ <i>ompT hsdS_B</i> (r _B ⁻ m _B ⁻) <i>gal dem</i> (DE3) pLysS	Novagen, Germany
BL1430	BL21(DE3)pLysS harboring pET1430, Kan ^r .	This work
<i>Xcc</i>		
8004	Wild-type, Rif ^r	(20)
1430nk	As 8004, but XC1430 (<i>zur</i> ::pK18mob, Rif ^r , Kan ^r)	(11)
2976nk	As 8004, but XC2976::pK18mob, Rif ^r , Kan ^r	This work
C2976nk	2976nk harboring pXC2976, Rif ^r , Kan ^r , Tc ^r	This work
wt/pG0267	8004 containing plasmid pG0267, Rif ^r , Tc ^r	This work
<i>zurmt</i> /pG0267	1430nk containing plasmid pG0267, Rif ^r , Kan ^r , Tc ^r	This work
wt/pG2471-2	8004 containing plasmid pG2471-2, Rif ^r , Tc ^r	This work
<i>zurmt</i> /pG2471-2	1430nk containing plasmid pG2471-2, Rif ^r , Kan ^r , Tc ^r	This work
wt/pG2976	8004 containing plasmid pG2976, Rif ^r , Tc ^r	This work
<i>zurmt</i> /pG2976	1430nk containing plasmid pG2976, Rif ^r , Kan ^r , Tc ^r	This work
wt/pG3788	8004 containing plasmid pG3788, Rif ^r , Tc ^r	This work
<i>zurmt</i> /pG3788	1430nk containing plasmid pG3788, Rif ^r , Kan ^r , Tc ^r	This work
wt/pG2976MT	8004 containing plasmid pG2976MT, Rif ^r , Tc ^r	This work
Plasmids		
pET-30a-C(+)	Expression vector, allow the production of fusion proteins containing amino terminal 6xHis-tagged sequences. Kan ^r	Novagen, Germany
pET1430	pET-30a-C(+)-containing the coding region of the <i>Xcc zur</i> gene. Kan ^r	This work
pLAFR6	Broad host range cloning vector, Tc ^r	(45)
pRK2073	Helper plasmid, Tra ⁺ , Mob ⁺ , ColE1, Spc ^r .	(46)
pG0267	pLAFR6 containing a XC0267 promoter- <i>gusA</i> fusion fragment, Tc ^r	This work
pG2471-2	pLAFR6 containing a XC2472-XC2471 promoter- <i>gusA</i> fusion fragment, Tc ^r	This work
pG2976	pLAFR6 containing a XC2976 promoter- <i>gusA</i> fusion fragment, Tc ^r	This work
pG3788	pLAFR6 containing a XC3788 promoter- <i>gusA</i> fusion fragment, Tc ^r	This work
pG2976MT	pLAFR6 containing a mutant XC2976 promoter- <i>gusA</i> fusion fragment, Tc ^r	This work
pXC2976	pLAFR6 containing a 1349-bp fragment including <i>XC2976</i> gene, Tc ^r	This work

^aRif^r, Kan^r, Spc^r, Tc^r = rifampicin-, kanamycin-, spectinomycin-, and tetracycline-resistant, respectively.

DNA ligase were used in accordance with the manufacturer's (Promega, Shanghai, China) instructions.

Construction of the DNA microarray

The genome of the *Xcc* strain 8004 (GenBank accession numbers CP000050) is composed of 4273 open reading frames (ORFs) (23). A DNA microarray encompassing 4186 ORFs of the strain 8004 has been constructed previously as described by He *et al.* (24) and used in this study.

RNA isolation and cDNA synthesis

For RNA isolation, 2 ml overnight culture of the wild-type strain 8004 or the *zur* mutant 1430nk was diluted into 100 ml of NYG medium and grown at 28°C with shaking at 200 r.p.m. When the cells reached an optical density at 600 nm of 1.0, cells were collected by centrifugation for 2 min at 12 000 r.p.m. The cells were resuspended in 10 ml TE (10 mM Tris-HCl, 1 mM EDTA) (pH 8.0), and frozen in a liquid nitrogen bath for 15 min. Total RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA, USA), and the contaminated DNA was removed by DNaseI (Promega) treatment. RNA integrity was confirmed by electrophoresis using a 1.3% formaldehyde-agarose gel, and the RNA quality was monitored by reverse transcription polymerase chain reaction (RT-PCR) analysis of two known genes. Three RNA samples were prepared from triplicate cultures of the wild-type strain 8004 and the *zur* mutant 1430nk, and used for cDNA synthesis or stored at -80°C until further use.

The RNAs isolated from the wild-type strain 8004 and the *zur* mutant 1430nk were used to make both Cy3- and Cy5-labeled cDNAs by reverse transcription and Klenow enzyme reaction. Briefly, 20 µg of total RNA and a series of dilutions (from 1 ng to 20 pg) of yeast intergenic spike RNA were utilized in reverse transcription reaction using 3 µg nonamer random primer and 200 units of M-MLV transcriptase (Invitrogen) in 1× first buffer [5 mM Tris-HCl (pH 8.3), 1 mM MgCl₂, and 7.5 mM KCl] at 37°C for 1 h. After the completion of the reaction, RNA was hydrolyzed by adding 5 µl of stop buffer (35 mM EDTA, 1.4 M NaOH) and neutralized with 1 µl of 10 N acetic acid. cDNA was purified with PCR Clean-up NucleoSpin Extract II kits (Macherey-Nagel, Düren, Germany) and dry vacuumed.

Slide hybridization and data analysis

For expression profiling hybridization, the labeled control and test cDNA samples were quantitatively adjusted according to the efficiency of Cy-dye incorporation, and then mixed with 80 µl hybridization solution (3×SSC, 0.2% SDS, 50% formamide). Prior to loading on the microarray, the mixture was heated to 95°C for 3 min to denature the cDNA in hybridization solution. Hybridization was performed under LifterSlip™ (Erie Company, Portsmouth, NH, USA), which allows for an even dispersal of hybridization solutions between the microarray and coverslip. The hybridization chamber was laid on a Three-phase Tiling Agitator (CapitalBio Corp., Beijing, China) to prompt the microfluidic circulation under the coverslip. The array was hybridized at 42°C overnight and washed

with two consecutive washing solutions (0.2% SDS, 2×SSC) at 42°C for 5 min and 0.2% SSC for 5 min at room temperature.

Arrays were scanned with a confocal LuxScan™ scanner (CapitalBio Corp., Beijing, China), and signal intensities were detected and quantified with SpotData software (CapitalBio Corp.) and assembled into EXCEL spreadsheets. Expression ratios were calculated as *zur*-mutant/wild-type (*zur*/WT), and the mean, SD and the statistical significance of the expression difference were calculated following the published method (25). Measurements with high variability, those where the standard deviation was equal to or larger than the mean, or those where valid ratios were found for only one of the three replicates, were removed. Three biological replicates of array hybridizations were performed and the data presented were the means of the three replicates.

Construction of an *E. coli* strain for production of the *Xcc* His₆-Zur protein

A 516-bp PCR product containing the coding region of the *Xcc zur* gene was amplified using the total DNA of the *Xcc* wild-type strain 8004 as template and the primer pair 1430PF/1430PR (Table S1) designed according to the sequence of the Zur-encoded ORF XC1430 of the strain 8004 (11,23). After confirmation by sequencing, the amplified DNA fragment was inserted into the BamHI and HindIII sites of the expression vector pET-30a-C(+) (Novagen, Darmstadt, Germany) to generate the recombinant plasmid pET1430 (Table 1), and the plasmid pET1430 was introduced into the *E. coli* strain BL21(DE3)pLysS (Novagen) to create the strain BL1430 (Table 1), which was used for (His)₆-Zur protein production.

Xcc His₆-Zur protein purification

Ten milliliters of overnight culture of strain BL1430 was used to inoculate a 200 ml LB medium with kanamycin and chloramphenicol and cultured at 37°C with shaking at 200 r.p.m. When the cell concentration reached OD₆₀₀ = 0.6, IPTG (isopropyl-beta-D-thiogalactopyranoside) was added to the culture at a final concentration of 1 mM, and the culture was further incubated at 37°C for 4 h. Cells were harvested by centrifugation at 4°C for 20 min at 4000 r.p.m. One gram of cells (wet weight) was resuspended in 4 ml lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole) and cells were ultrasonicated for 30 min. The cell lysate was centrifuged at 4000g for 20 min at 4°C, and the clear supernatant was transferred into a 50 ml centrifugation tube and mixed with 2.5 ml of prewashed Ni-NTA resin (Qiagen, Hilden, Germany) in the lysis buffer. After shaking with 200 r.p.m. at 4°C for 2 h, the batch was poured into a plastic Ni-NTA Spin Column (Qiagen) and then washed several times with 50 ml of precooled wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole) until the absorbance at 280 nm was <0.01. Finally, the (His)₆-Zur protein was eluted with 500 µl of precooled elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole) and was analyzed by SDS-PAGE or stored at -20°C.

Construction of reporter plasmids

To construct reporter plasmids for the ORFs *XC0267*, *XC2471-2* (*XC2471* and *XC2472*), *XC2976* and *XC3788*, the 150–300 bp region upstream of the start codon (excluding the start codon) of the genes was respectively amplified by PCR using the total DNA of the *Xcc* wild-type strain 8004 as template and the primer pairs 0267PF/0267PR, 2471-2PF/2471-2PR, 2976PF/2976PR and 3788PF/3788PR (Table S1). To ensure these promoter fragments could be ligated with the promoterless *gusA* (β -glucuronidase) gene fragment by fusion PCR (26), a 20-nt tag complementary to the first 20 nt of the promoterless *gusA* fragment was added to the 5'-end of the primers 0267PR, 2471-2PR, 2976PR and 3788PR (Table S1). A 1.8-kb DNA fragment containing the promoterless *gusA* gene with its RBS (ribosomal-binding site) was amplified by PCR using pLAFR1::Tn5*gusA5* as template and the primer pair GusAF/GusAR (Table S1). Each of the amplified promoter fragments and the 1.8-kb *gusA* fragment were respectively ligated by fusion PCR to generate the promoter-*gusA* fusion fragments. Then, these fusion fragments were respectively cloned into the broad-host-range vector pLAFR6 to create the reporter plasmids pG0267, pG2471-2, pG2976 and pG3788 (Table 1). These reporter plasmids were respectively introduced into the wild-type strain 8004 to create the strains wt/pG0267, wt/pG2471-2, wt/pG2976 and wt/pG3788, and introduced into the *zur* mutant 1430nk to create the strains *zurmt*/pG0267, *zurmt*/pG2471-2, *zurmt*/pG2976 and *zurmt*/pG3788 (Table 1).

Site-directed mutagenesis of the *XC2976* promoter

To mutate the *XC2976* promoter, ACACCACACC was changed for its left half part CGTGATGTGA of the 20-bp inverted repeat. Firstly, a 130- and 1956-bp DNA fragments were respectively amplified by PCR using the *XC2976*-promoter-containing reporter plasmid pG2976 (see above) as template and the primer sets 2976PF/2976MTR and 2976MTF/GusAR (Table S1). To ensure the two fragments could be ligated together by fusion PCR, the first 20-nt sequence of the 5'-end of the two primers were designed to complementary each other (Table S1). To obtain the expected nucleotide changes, the sequences of the primers 2976MTR and 2976MTF were designed to contain desired mutant sequence (Table S1). After confirmed by sequencing, the two DNA fragments were ligated by fusion PCR to generate the mutant *XC2976* promoter-*gusA* fusion fragment. Then, the fusion fragment was cloned into the broad-host-range vector pLAFR6 to create the reporter plasmid pG2976MT (Table 1). The plasmid pG2976MT was introduced into the wild-type strain 8004 to create the strain wt/pG2976MT (Table 1).

The mutated *XC2976* promoter fragment P_{2976MT}, in which ACACCACACC was changed for the left half part of the 20 bp imperfect inverted repeat CGTGATGTGA of the wild-type *XC2976* promoter, was obtained by PCR using pG2976MT DNA as template and the primer set P_{2976-F}/P_{2976-R} (Table S1).

GUS activity assay

The activity of β -glucuronidase (GUS) was determined after the growth of *Xcc* strains in NYG, NYG supplemented with different levels of ZnSO₄ or EGTA for 24 h by measurement of the OD₄₁₅ using *p*-nitrophenyl β -D-glucuronide as the substrate, as described by Jefferson *et al.* (27).

Electrophoretic mobility shift assay (EMSA)

EMSA was performed following the procedure described by Gaballa and Helmann (6). Fragments containing the promoter regions of the ORFs *XC0267*, *XC2471-2*, *XC2976* and *XC3788* or the intergenic region between *XC2471* and *XC2472* (IGR₂₄₇₁₋₂₄₇₂, as a negative control) were amplified by PCR using the total DNA of the wild-type strain 8004 as the template and the primer pairs P_{0267-F}/P_{0267-R}, P_{2471-2-F}/P_{2471-2-R}, P_{2976-F}/P_{2976-R}, P_{3788-F}/P_{3788-R} and IGR_{2471-2-F}/IGR_{2471-2-R} (Table S1). After purified by using QIAquick PCR Purification Kit (Qiagen), these PCR products were labeled with [γ -³²P]ATP using a 5'-end labeled kit (Takara, Dalian, China). Then the His₆-Zur protein was added into a 1.5-ml EP tube containing 20 μ l binding buffer [20 mM Tris-HCl (pH 8), 50 mM KCl, 1 mM DTT, 5% glycerol, 0.1 mg of bovine serum albumin per ml, and 5 μ g of sheared salmon sperm DNA per ml] to final concentrations of 0, 8, 16, 32, 64, 128, 256 and 508 nM, and 3 pmol labeled DNA was added to each tube and mixed well. The mixtures were incubated at room temperature for 20 min. Samples were then loaded on a 4% polyacrylamide gel prepared and run in 40 mM Tris-acetate buffer (with no EDTA) (pH 8.0). The gel was dried and exposed to a phosphorimager screen (Typhoon 9410, Amersham Biosciences Corp., Piscataway, NJ USA).

To test the effects of Zn²⁺ and EDTA on protein-DNA binding, the His₆-Zur protein was added into 20 μ l binding buffer and then ZnSO₄ or EDTA was added and mixed well. After incubation for 10 min at room temperature, 1–3 pmol labeled DNA was added and mixed well, and the mixture was incubated at room temperature for 20 min. Samples were then analyzed as described above.

DNaseI footprinting analyses

DNaseI footprinting to map the binding site of Zur in the promoter region of the ORFs *XC0267*, *XC2471-2*, *XC2976* and *XC3788* was performed using fluorescently labeled DNA and the ABI 377 DNA sequencer (Applied Biosystems, Foster City, CA, USA) to resolve the digestion products, as described by Merighi *et al.* (28). The same primer sets (Table S1) used for amplification of the DNA fragments for EMSA, which contain respectively the promoter region of the ORFs *XC0267*, *XC2471-2*, *XC2976* and *XC3788*, were used to amplify the corresponding promoter fragments for DNaseI footprinting analyses. The 5'-ends of the primers P_{0267-R}, P_{2471-2-R}, P_{3788-R} and P_{2976-F} were labeled with 5-carboxytetramethylrhodamine (Tamra) (Sangon, Shangshai, China). DNA fragments containing the promoter region of the ORFs *XC0267*, *XC2471-2*, *XC2976* and *XC3788* were

amplified as a 5'-end-labeled PCR product using the total DNA of the wild-type strain 8004 as template. The PCR products were purified by gel electrophoresis and quantified using UV spectrophotometer. The labeled probes (400 ng) were incubated with 120 ng of His₆-Zur protein in 20 µl binding buffer [20 mM Tris-HCl (pH 8), 50 mM KCl, 1 mM DTT, 5% glycerol, 0.1 mg of bovine serum albumin per ml, and 5 µg of sheared salmon sperm DNA per ml] at 28°C for 20 min, immediately prior to use, 5 µl RQ1 DNaseI (1 U/µl) was diluted in 100 µl cold Tris-HCl (10 mM, pH 8.0), and then 5 µl DNaseI (Promega) was added to the 20-µl reaction mixture and incubated for 1 min. The reaction was stopped by addition of 5 µl of 0.15M EDTA, 5% SDS and extracted with phenol-chloroform-isoamyl alcohol (25:24:1). The DNA fragments were purified in a Qiagen spin column. Sequencing of the corresponding nonlabeled promoter fragments were performed using ABI BigDye Terminator 2.0 sequencing kit (Applied Biosystems) according to the manufacturer's instructions. Sequencing reactions contained 100 ng of promoter fragment DNA and 3.2 pmol of nonlabeled P₀₂₆₇-R, P₂₄₇₁₋₂-R, P₃₇₈₈-R and P₂₉₇₆-F primer. A 1-µl aliquot of sequencing product and DNaseI digestion product was loaded onto an ABI 377 DNA sequencer (Applied Biosystems) for a 'Sequencing running'. Photos of the 'Gel Image' were directly taken from the screen of the sequencer. To determine the size of the DNaseI digestion fragments, a 0.1-µl aliquot of Genescan-350 size standard (Applied Biosystems) was combined with either 1 µl sequencing product or 1 µl DNaseI digestion product and loaded onto an ABI 377 DNA sequencer for a 'Genescan running'. Electropherograms of the Genescan running were analyzed using Genescan 3.1 (Applied Biosystems).

Rapid amplification of cDNA end (5' RACE)

RNA was extracted from the wild-type strain 8004 grown to the mid-exponential phase (OD₆₀₀ ≈ 0.6) in the rich medium NYG using the SV Total RNA Isolation System (Promega), and treated with DNaseI (Promega). Five micrograms of the DNA-free RNA and 15 pmol of the gene specific primers GSP1s (Table S1) were incubated at 70°C for 5 min and then 42°C for 1 h in the presence of 1 × M-MuLV-RT buffer, 1 mM dNTPs, 20 U RNase inhibitor (Fermentas, Burlington, Canada) and 200 U M-MuLV Reverse Transcriptase (Fermentas). After treated with 2 U/µl RnaseH (Promega) for 30 min (to remove the remaining RNAs), the reaction product (cDNA) was purified using S.N.A.P.Column (Invitrogen) and finally resolved in 50 µl sterilized dH₂O. Then, 10 µl purified cDNA was incubated at 37°C for 10 min in the presence of 0.2 mM dCTP and 20 U of TDT (terminal deoxynucleotidyl transferase) (Invitrogen) to add a poly(C) tail to the 3'-end of the cDNA. Finally, 5 µl poly(C) tailed cDNA was used as template for PCR reaction. To improve assay sensitivity, two rounds of semi-nested PCR were performed. The first semi-nested PCR was performed using 5 µl poly(C) tailed cDNA as template, and AAP and an internal oligonucleotide, GSP2, as primer (Table S1); the second semi-nested PCR was performed using the product

of the first semi-nested PCR as template, and AUAP and another internal oligonucleotide, GSP3, as primer (Table S1). After gel purification, the last PCR product was sequenced using GSP3 as primer.

In vitro transcription assays

In vitro transcription assays on the promoter DNA of *XC0267*, *XC2471-2*, *XC2976* and *XC3788* were performed using the experimental procedure described by Friedman *et al.* (29). The promoter template DNA fragments of *XC0267* (P₀₂₆₇), *XC2471-2* (P₂₄₇₁₋₂), *XC2976* (P₂₉₇₆) and *XC3788* (P₃₇₈₈) were respectively generated by PCR amplification using the total DNA of the wild-type strain 8004 as template and primer sets 0267ivtF/0267ivtR, 2471-2ivtF/2471-2ivtR, 2976ivtF/2976ivtR and 3788ivtF/3788ivtR (Table S1). To generate P_{2976MT}, a template DNA fragment containing a mutated *XC2976* promoter in which the 20 bp imperfect inverted repeat sequence is lacking, fragment A and fragment B were respectively amplified using the total DNA of strain 8004 as template and primer sets 2976PF/2976MTR and 2976MTF/2976ivtR (Table S1), and fragments A and B were then linked together to generate the P_{2976MT} fragment by fusion PCR (26). Each promoter template fragment includes the promoter region as well as a ~150-bp coding region downstream of the annotated translation start site of the corresponding gene (23). To remove imidazole, Zur was dialyzed against 200 volumes of Tris-HCl buffer [10 mM Tris-HCl (pH 8.0), 1 mM DTT] at 4°C. For *in vitro* transcription, Zur was incubated for 30 min at room temperature in transcription buffer [40 mM Tris-HCl (pH 7.9), 6 mM MgCl₂, 2 mM spermidine, 10 mM NaCl, 5 mM DTT, 5% glycerol, 50 mM KCl, 1U RNase inhibitor, 100 µM ZnSO₄] containing 4 nM promoter template DNA. Then a NTP mixture [250 µM each of ATP, CTP and GTP, 20 µM UTP, 8 µM (α-³²P)UTP (3000 Ci/mmol, 10 mCi/ml)] and 1 U *E. coli* RNA polymerase Holoenzyme (sigma saturated) (Epicentre, Madison, WI, USA) was added to start the transcription. After incubation at 28°C for 30 min, reactions were terminated by addition of 1 volume of 2× Loading Dye Solution (Fermentas) and chilled on ice. After incubation at 70°C for 10 min, transcription products were run on a 5% denatured polyacrylamide gel containing 7M urea in 1× Tris borate-EDTA electrophoresis buffer. The transcripts obtained were analyzed by a phosphorimager screen (Typhoon 9410, Amersham Biosciences, USA). Each experiment was repeated three times.

Mutant construction

A mutant of *XC2976* was constructed by homologous suicide plasmid integration (30) using pK18mob as the vector (31). A 380-bp internal fragment of *XC2976* was amplified using the total DNA of the *Xcc* wild-type strain 8004 as the template and the primer pair 2976MF/2976MR (Table S1). After confirmation by sequencing, the amplified DNA fragment was cloned into the suicide plasmid pK18mob to create the recombinant plasmid pK2976 (Table 1). The plasmid was transformed from *E. coli* JM109 (32) into *Xcc* strain 8004 by triparental

conjugation using pRK2073 as the helper plasmid. Transconjugants were screened on NYG supplemented with rifampicin and kanamycin and the obtained transconjugants with a mutation in *XC2976* were confirmed by PCR. Confirmation PCR was performed using the total DNA of the transconjugants as the template along with the primer pair P18conF/2976conR (Table S1), and the total DNA of the *Xcc* wild-type strain 8004 was used as a negative control. The primer P18conF is located in pK18mob, and 2976conR is located downstream of the cloned internal fragment of *XC2976*. The expected PCR products were further confirmed by sequencing. One of the confirmed mutant transconjugants was designated as 2976nk, and was chosen for further study (Table 1).

Complementation of the mutant 2976nk

For complementation of the *XC2976* mutant 2976nk, a 1349-bp DNA fragment containing the entire *XC2976* gene (from 200-bp upstream of the start codon to 182-bp downstream of the stop codon) was amplified by PCR using the total DNA of the *Xcc* wild-type strain 8004 as the template and the primer pair 2976CF/2976CR (Table S1). After confirmation by sequencing, the amplified DNA fragment was cloned into pLAFR6 to generate the recombinant plasmid pXC2976 (Table 1). The plasmid pXC2976 was transferred into the mutant 2976nk by tri-parental conjugation. The transconjugants carrying pXC2976 were screened on NYG with rifampicin, kanamycin and tetracycline. A confirmed transconjugant representative was named C2976nk (Table 1) and chosen for further study.

Metal ion sensitivity test

For metal ion sensitivity test, 200- μ l overnight culture of *Xcc* strains with optical density at 600 nm of 1.0 ($OD_{600} = 1.0$) was inoculated into 200-ml NYG medium supplemented with a certain metal ion to a series of final concentrations, and the cell density for each treatment was measured spectrometrically at 600 nm after incubated at 28°C with shaking at 200 r.p.m. for 24 h. The levels of metal ions used in this study are $ZnSO_4$: 0, 300, 400 and 500 μ M; $CoCl_2$: 0, 150, 200, 250, 300 and 350 μ M; $CdSO_4$: 0, 40, 50, 60, 70 and 80 μ M and $NiSO_4$: 0, 400, 500, 600, 700 and 800 μ M.

Measurement of Zn^{2+} content in cells

Two hundred microliters of overnight culture of *Xcc* strains were inoculated into 200-ml NYG medium. After incubation at 28°C with shaking at 200 r.p.m. for 12 h, the culture was added with $ZnSO_4$ to a final concentration of 300 μ M and incubated for further 4 h. Cells from the culture were harvested and washed twice with 0.1 M LiCl, 0.2 mM EDTA and 0.1 mM EGTA to remove externally bound metal ions. The cell density was adjusted using sterilized ddH₂O to an OD_{600} of 1.0 and the cells were kept on ice. The Zn^{2+} content in the cells was determined by an atomic absorption spectrophotometer with a HITACHI Model 2000 instrument.

RESULTS

Xcc Zur negatively regulates three genes related to Zn^{2+} -uptake systems and positively regulates a gene related to a Zn^{2+} efflux pump

Our previous work showed that in *Xcc* inactivation of *zur* resulted in hypersensitivity to Zn^{2+} toxicity (11), indicating that certain important genes involved in Zn^{2+} homeostasis of *Xcc* are under the control of Zur. To identify these genes, we screened putative Zur-regulated genes by genome-wide DNA microarray hybridization. RNAs were isolated from the cells of the wild-type *Xcc* strain 8004 and the *Xcc zur* mutant 1430nk (Table 1) grown in the Zn^{2+} -replete medium NYG (ca. 10 μ M Zn^{2+}) (11), and used to make both Cy3- and Cy5-labeled cDNAs. The labeled cDNAs were hybridized with a previously constructed DNA microarray encompassing 4186 of the 4273 total ORFs of the wild-type strain 8004 (23,24), and the *zur* mutant/wild-type expression ratios were compared (see Materials and methods section for details). The results showed that 37 and 27 ORFs displayed statistically significant (*t*-test, $P < 0.05$) increases and decreases greater than 2-fold in mRNA levels by *zur* mutation, respectively (Table S2), suggesting that these 64 ORFs may be regulated by Zur.

As shown in Table S2, none of the 64 putative Zur-regulated genes is annotated to have a metal ion homeostasis-related function except *XC2976*, which was predicted to encode a cobalt–zinc–cadmium resistant protein (23) showing 67% amino acid sequence similarity to the Zn^{2+} efflux pump CzcD of *Ralstonia metallidurans* (33). However, when we used the amino acid sequences of the predicted products of these putative Zur-regulated genes to blast (34) the NCBI database (<http://www.ncbi.nlm.nih.gov/blast>), in addition to *XC2976*, the predicted products encoded by *XC0267*, *XC2471* and *XC3788* displayed amino acid sequence similarity to Zn^{2+} homeostasis-related proteins identified in other bacteria. *XC2471* and *XC0267* showed 29 and 54% amino acid sequence similarities to YciA and YciC of *B. subtilis*, respectively, while *XC3788* exhibited 38% similarity to ZnuC of *E. coli* (Table 2). YciA and YciC are components of the low-affinity Zn^{2+} -uptake system YciABC of *B. subtilis* (16) and ZnuC is a member of the high-affinity Zn^{2+} -uptake system ZnuABC of *E. coli* (5). Based on these similarities, we propose that *XC2976* is a putative Zn^{2+} efflux-related gene and *XC0267*, *XC2471* as well as *XC3788* are putative Zn^{2+} uptake-related genes. Interestingly, a survey using the amino acid sequences of the *E. coli* ZnuA and ZnuB to blast the genome of the *Xcc* strain 8004 (23) exhibited no homologous sequences (data not shown).

The locations of the putative Zn^{2+} homeostasis-related genes *XC0267*, *XC2471*, *XC3788* and *XC2976* in the genome of *Xcc* strain 8004 are shown in Table 2. *XC0267* and its adjacent ORF *XC0266* are separated by a spacer with only 61 bp and their transcriptions are back to back (Figure 1). The ORFs *XC2471* and *XC2472* share the same transcriptional direction and are separated by only 34 bp (Figure 1). To determine whether these two ORFs are transcribed together, we performed

Table 2. Similarity of the products of genes that showed altered expression in the *zur* mutant to characterized bacterial metal ion homeostasis-related proteins

ORFs	Positions in <i>Xcc</i> 8004's genome	Homology [Organism] (accession number)	Amino acid similarity (%)	Characterized function	Reference
XC0267	321454–322779	YciC [<i>B.subtilis</i>](CAB12130)	54	Component of the low-affinity Zn ²⁺ uptake system YciABC	(16)
XC2471	2989376–2988441	YciA [<i>B. subtilis</i>] (CAB12128)	29	Component of the low-affinity Zn ²⁺ uptake system YciABC	(16)
XC2976	3563238–3562273	CzcD [<i>R. metallidurans</i>] (YP_145596)	67	Acts as a Zn ²⁺ efflux pump	(33)
XC3788	4483267–4482527	ZnuC [<i>E. coli</i>] (P0A9X1)	38	Component of the high-affinity Zn ²⁺ uptake system ZnuABC	(5)

a RT-PCR using the cDNA converted from the RNA of the wild-type strain 8004 as template and the primers 2471-2RTF/2471-2RTR (Table S1). An amplified DNA fragment with 386 bp in length was obtained (Figure 1; data not shown), indicating that the ORFs *XC2471* and *XC2472* are together transcribed and they may share the same promoter located in the upstream region of *XC2472* (Figure 1). Therefore, we designated the promoter and reporter plasmid of these two ORFs as P_{XC2471-2} and pG2471-2, respectively (see below). To establish the genetic relationship between *zur* and *XC0267*, *XC2471*, *XC2976* as well as *XC3788* we constructed plasmid-driven promoter-*gusA* transcriptional fusion reporters of these genes by fusing the promoterless *gusA* gene with the promoter of the genes and cloning the fused fragment into the plasmid pLAFR6, respectively. The obtained reporter plasmids were named pG0267, pG2471-2, pG2976 and pG3788, respectively (Table 1; see Materials and methods section for details). These reporter plasmids were introduced into the wild-type strain 8004 (wt) and the *zur* mutant strain 1430nk (*zurmt*) to yield reporter strains wt/pG0267, wt/pG2471-2, wt/pG2976, wt/pG3788, *zurmt*/pG0267, *zurmt*/pG2471-2, *zurmt*/pG2976 and *zurmt*/pG3788, respectively (Table 1). The GUS activities of these strains were determined in Zn²⁺-rich conditions (see Materials and methods section for details). As shown in Table 3, the GUS activities of the reporter plasmids pG0267, pG2471-2 and pG3788 under the wild-type background are respectively 15-, 5- and 31-fold lower than those under the *zur* mutation background; in contrast, the GUS activity of pG2976 under the wild-type background is ~9-fold higher than that under the *zur* mutation background. The differences are statistically significant ($P = 0.01$ by *t*-test). These results demonstrate that the three putative Zn²⁺-uptake genes *XC0267*, *XC2471*, and *XC3788* are negatively regulated while the putative Zn²⁺ efflux gene *XC2976* is positively regulated by Zur.

The expression of *XC0267*, *XC2471-2*, *XC2976* and *XC3788* responses to Zn²⁺ through the mediation of Zur

To determine whether the expression of *XC0267*, *XC2471-2* (*XC2471* and *XC2472*), *XC2976* and *XC3788* is affected by Zn²⁺ concentrations, the GUS activities of the reporter strains wt/pG0267, wt/pG2471-2, wt/pG2976 and wt/pG3788 in the Zn²⁺-rich conditions were compared to those in the Zn²⁺-deficient conditions.

The results showed that when the Zn²⁺ chelator EGTA was added into the Zn²⁺-rich NYG medium, the GUS activities of the reporter strains wt/pG0267, wt/pG2471-2 and wt/pG3788 were about 3-, 4- and 2-fold increased, respectively, while the GUS activity of wt/pG2976 was about 2-fold decreased (Table 3). Statistical analysis revealed that these differences are significant ($P = 0.01$ by *t*-test). These altered GUS activities were due to the change of the free Zn²⁺ in the medium, because addition of ZnSO₄ into the EGTA-supplemented NYG medium to a final concentration of 0.2 mM could restore the GUS activities of all the reporter strains to the levels in the Zn²⁺-rich NYG medium (Table 3). The results demonstrate that the expression of *XC0267*, *XC2471-2* and *XC3788* are repressed in Zn²⁺-rich condition and induced in Zn²⁺-limited condition. On the contrary, *XC2976* is induced in Zn²⁺-rich condition and repressed in Zn²⁺-limited condition. These further support the possibility that *XC0267*, *XC2471* and *XC3788* may play roles in Zn²⁺ uptake-related function and *XC2976* in Zn²⁺ export-related function.

The GUS activities of the reporter strains wt/pG0267, wt/pG2471-2 and wt/pG3788 in NYG medium with 0.5 mM EGTA are significantly lower than those of *zurmt*/pG0267, *zurmt*/pG2471-2 and *zurmt*/pG3788 in NYG medium without addition of EGTA, respectively (Table 3), indicating that addition of 0.5 mM EGTA could not completely release the repressing effect of Zur. Increasing EGTA concentration did not lead to a further increase of the GUS activities of these reporter strains (data not shown). Furthermore, the GUS activities of the reporter strains *zurmt*/pG0267, *zurmt*/pG2471-2, *zurmt*/pG3788 and *zurmt*/pG2976 were not affected by the Zn²⁺ status in the NYG medium (Table 3). These results suggest that the Zn²⁺ repressing effects on the expression of *XC0267*, *XC2471-2* as well as *XC3788* and the inducing effects on the expression of *XC2976* are mediated by Zur.

Zur activates the transcription of *XC2976* and represses *XC0267*, *XC2471-2* and *XC3788* directly

The above studies have demonstrated that the *Xcc* Zur positively regulates the expression of *XC2976* and negatively regulates the expression of *XC0267*, *XC2471-2* and *XC3788*. To investigate whether Zur directly regulates these genes, we performed EMSA assays to determine if

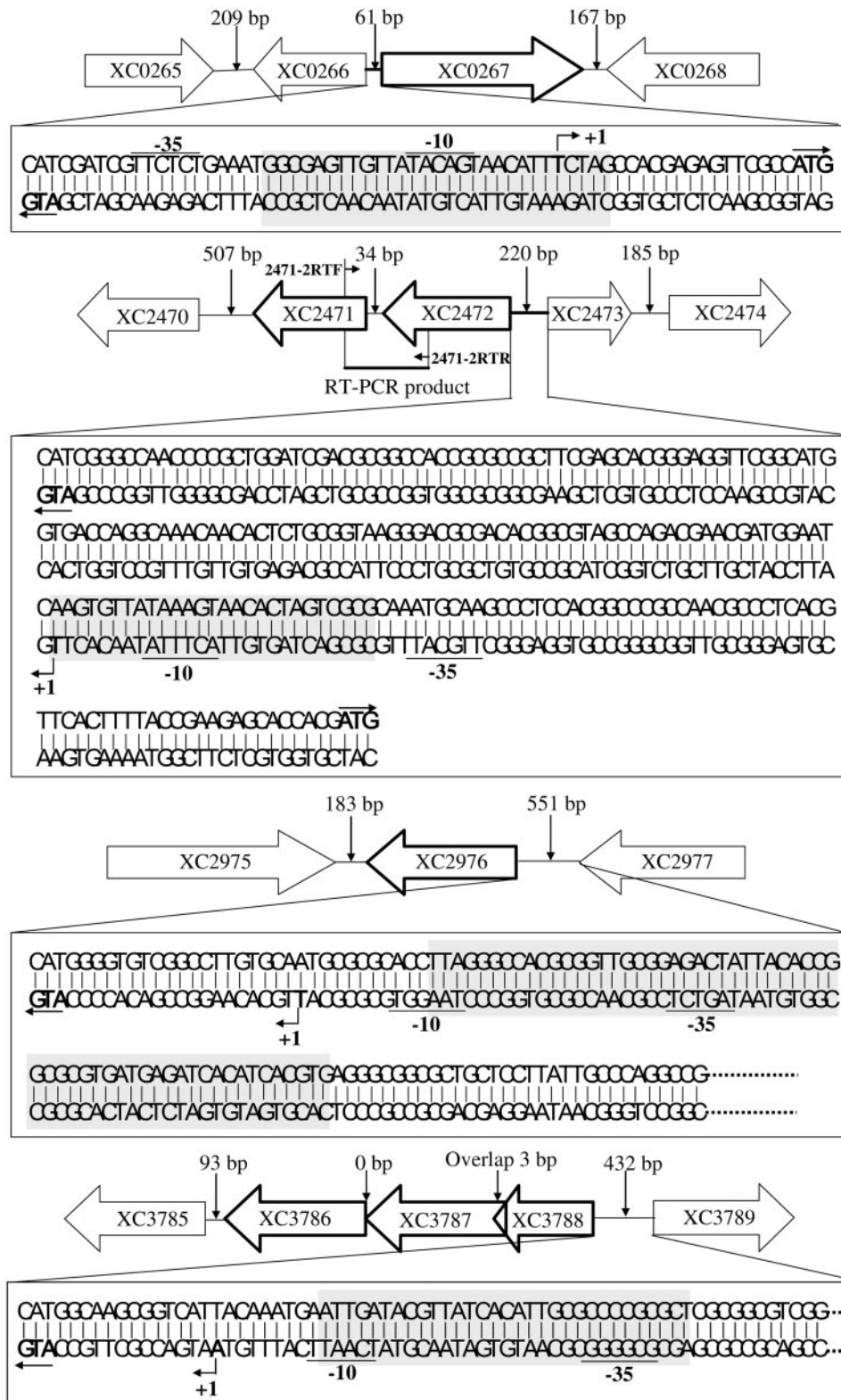


Figure 1. The genetic organization of the Zur-regulated genes and the detailed genetic elements in their promoters. The genetic organization of *XC0267*, *XC2471*, *XC2976* and *XC3788* loci was based on the genome sequencing data of the *Xcc* strain 8004 (23). The identified transcriptional start sites (+1) are shown, and the deduced -35 and -10 promoter regions are underlined. Gray boxes denote the position and sequence of the Zur-binding sites in promoter regions of the genes. Value above a vertical narrow denotes the length of the spacer between the nearest two ORFs.

Table 3. The GUS activity of different reporter strains in zinc-rich and zinc-deficient conditions

Reporter strain	GUS activity (U) ^a		
wt/pG0267	0.12 ± 0.02A	0.33 ± 0.01B	0.13 ± 0.03A
zurmt/pG0267	1.78 ± 0.01C	1.77 ± 0.11C	1.75 ± 0.13C
wt/pG2471-2	0.04 ± 0.01A	0.14 ± 0.02B	0.06 ± 0.03A
zurmt/pG2471-2	0.21 ± 0.03C	0.25 ± 0.02C	0.22 ± 0.02C
wt/pG3788	0.13 ± 0.01A	0.32 ± 0.03B	0.11 ± 0.02A
zurmt/pG3788	4.04 ± 0.34C	4.69 ± 0.17C	4.57 ± 0.02C
wt/pG2976	4.46 ± 0.10A	1.71 ± 0.21B	5.06 ± 0.23A
zurmt/pG2976	0.49 ± 0.01C	0.54 ± 0.02C	0.51 ± 0.06C

^a β -Glucuronidase (GUS) activities were respectively determined after the growth of *Xcc* strains in NYG, NYG supplemented with EGTA to the final concentration of 0.5 mM (NYG + EGTA) or NYG supplemented with EGTA and ZnSO₄ to the final concentrations of 0.5 mM and 0.2 mM (NYG + EGTA + Zn²⁺) for 24 h. Data are the mean ± SD of triplicate measurements. Each experiment was repeated three times and similar results were obtained. The different letters in each horizontal data column indicate significant differences at $P = 0.01$.

the *Xcc* Zur binds to the promoter region of these genes. The His₆-tagged Zur protein of *Xcc* was overexpressed and purified from *E. coli* (see Materials and methods section for details). The DNA fragments of the promoter regions (~200-bp upstream of the start codon) of the ORFs *XC0267*, *XC2471-2*, *XC2976* and *XC3788* were amplified by PCR using the total DNA of the *Xcc* wild-type strain 8004 as template and the primer sets P_{0267-F}/P_{0267-R}, P_{2471-2-F}/P_{2471-2-R}, P_{2976-F}/P_{2976-R} and P_{3788-F}/P_{3788-R} (Table S1) and designated as P₀₂₆₇, P₂₄₇₁₋₂, P₂₉₇₆ and P₃₇₈₈, respectively (see Materials and methods section for details). The binding ability of the His₆-Zur to these promoter-containing DNA fragments was evaluated by EMSA (see Materials and methods section). The EMSA results showed that the His₆-Zur could bind to P₀₂₆₇, P₂₄₇₁₋₂, P₂₉₇₆ and P₃₇₈₈ with a high affinity, but not to a DNA fragment containing the intergenic region of the ORFs *XC2471* and *XC2472* (IGR₂₄₇₁₋₂₄₇₂) (Figure 2A). The purified His₆-Mip-like protein (35) was unable to bind to the promoter fragments P₀₂₆₇, P₂₄₇₁₋₂, P₂₉₇₆ and P₃₇₈₈ (data not shown). These indicate that the Zur-DNA binding detected in the EMSA was specific and the His₆ tag did not interfere the binding. The results suggest that the *Xcc* Zur may directly regulate *XC0267*, *XC2471-2*, *XC2976* and *XC3788*.

To determine the role of Zn²⁺ in the binding of Zur with its target DNA, we compared the binding ability of Zur to the promoter fragments P₀₂₆₇, P₂₄₇₁₋₂, P₂₉₇₆ and P₃₇₈₈ in the binding mixtures with and without addition of the Zn²⁺ chelator EDTA. The EMSA results showed that addition of EDTA blocked Zur-DNA binding and further addition of Zn²⁺ into the EDTA-containing binding mixture could fully restore the DNA binding ability of Zur (Figure 2B). However, addition of Cu²⁺ could not return the binding ability (Figure 2B). These reveal that the binding of Zur with its DNA targets requires Zn²⁺ as a cofactor.

To further clarify whether Zur directly activates the transcription of *XC2976* and represses *XC0267*, *XC2471-2* and *XC3788*, *in vitro* transcription assays were performed on the promoter DNA of these genes (see Materials and methods section for details). The results

showed that addition of Zur to the *in vitro* reactions decreased the transcription from the promoters of *XC0267*, *XC2471-2* and *XC3788*, but increased the transcription from the *XC2976* promoter (Figure 3A–D). These demonstrate that Zur represses the transcription of *XC0267*, *XC2471-2* and *XC3788*, and activates the transcription of *XC2976*, directly.

Zur recognizes two distinct DNA targets

We employed DNaseI footprinting analysis to ascertain the Zur-binding sequences in its target promoters (see Materials and methods section for details). As shown in Figure 2C, the purified His₆-tagged Zur protein bound to a region of 59-bp in length in the promoter of *XC2976* and an about 30-bp region in the promoters of *XC0267*, *XC2471-2* and *XC3788*. Sequencing analysis displayed that the Zur protected regions in the promoters of *XC0267*, *XC2471-2* and *XC3788* are about 30-bp AT-rich sequences showing high similarity to the *E. coli* Zur-binding sequences (Zur-box) (AAGTGTGATATTATAACATTTTCATGACTA) (14,15) (Figure 2D). Multiple alignment of the Zur-protected sequences in the promoters of *XC0267*, *XC2471-2* and *XC3788* as well as the *E. coli* Zur box using the Vector NTI Alignment program (Invitrogen) revealed a conserved core sequence aagTGTg(t)ATAaagTAaCAtTtct (Figure 2D). Interestingly, the Zur-protected region in the *XC2976* promoter is a 59-bp GC-rich (57.7%) sequence (AATCCCGG TGCGCCAACGCCTCTGATAATGTGGCCGCGCAC TACTCTAGTGTAGTGCAC) containing a 20-bp imperfect inverted repeat, GCACTACTCT-AGTGTAGTGC. Sequence comparison exhibited that this inverted repeat sequence displays no significant similarity to the Zur-binding sequences in the promoters of *XC0267*, *XC2471-2* and *XC3788*. These results, taken together, suggest that the *Xcc* Zur can recognize two distinct DNA targets.

As shown in Figure 2C, five and one additional DNaseI hypersensitive bands were respectively observed in the Zur-protected regions of *XC0267* and *XC2471-2* promoters, suggesting that the *Xcc* Zur might induce local distortions in the target DNAs upon binding. However, no additional hypersensitive band was observed in the Zur-protected regions of *XC3788* and *XC2976* promoters (Figure 2C), indicating that the interactions of the *Xcc* Zur with these promoters might be somewhat different. Furthermore, the Zur-protected sequence on the *XC2976* promoter was approximately twice as wide as that on the promoters of the *XC0267*, *XC2471-2* and *XC3788*, suggesting that there might be more Zur molecules bound to the *XC2976* promoter. Consistent with this suggestion is the observation that the Zur-P₂₉₇₆ complex migrated more slowly than the Zur-P₀₂₆₇, Zur-P₂₄₇₁₋₂ and Zur-P₃₇₈₈ complexes (data not shown).

To clarify the Zur-binding site in the above Zur-regulated promoters is located within the –35 to –10 region or outside the region, the transcriptional start sites of *XC0267*, *XC2471-2*, *XC3788* and *XC2976* were determined by 5' RACE PCR (see Materials and methods section for details). After the transcriptional start site was determined, a potential –35 and –10 sequence in the

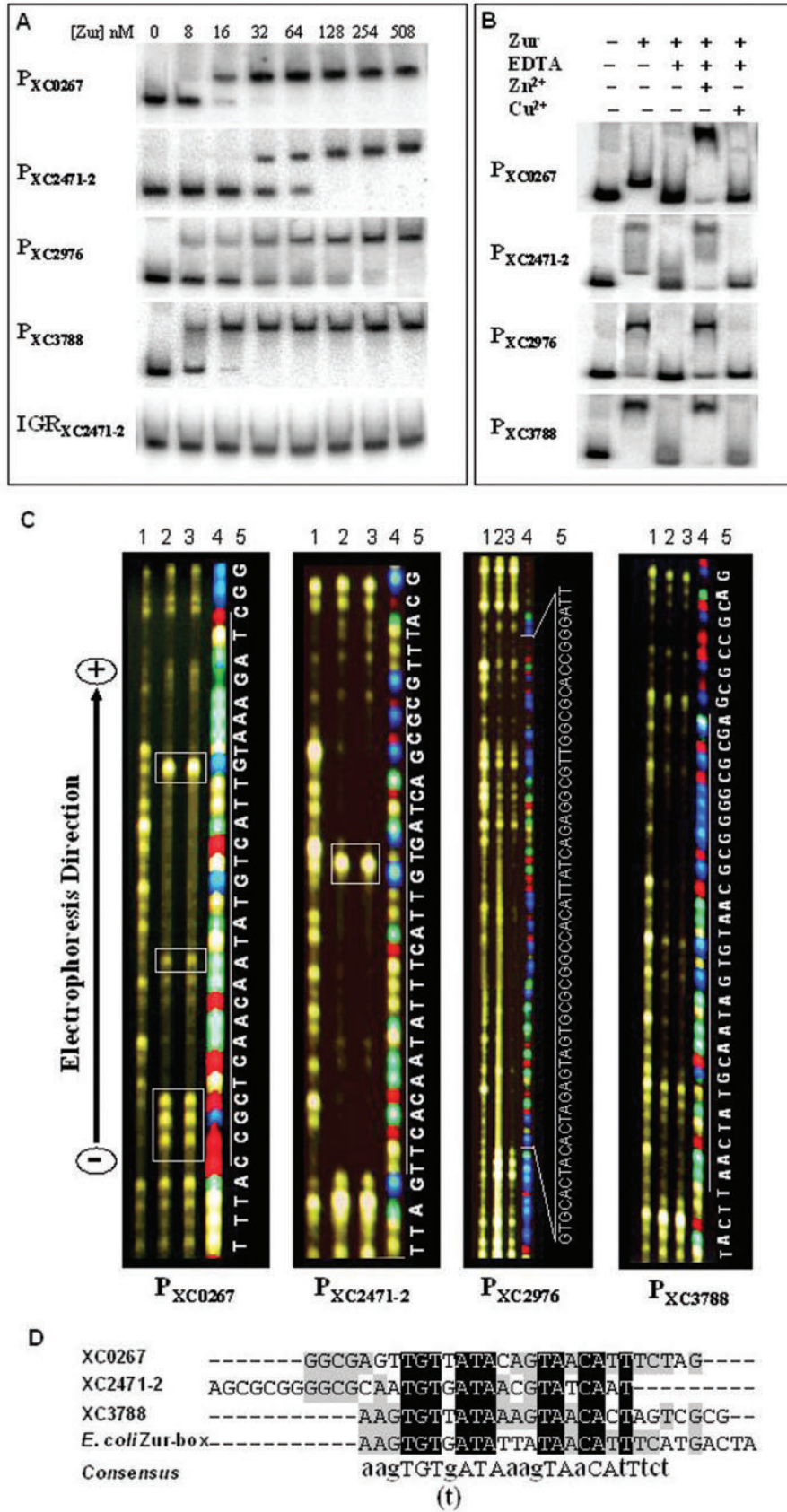


Figure 2. The interaction of Zur with the promoters of Zur-regulated genes. (A) EMSA of Zur binding to the promoter regions of XC0267 (P_{0267}), XC2471-2 (P_{2471-2}), XC2976 (P_{2976}) and XC3788 (P_{3788}). Zur protein was incubated with 3 pmol of ³²P-end-labeled DNA fragment in binding buffer

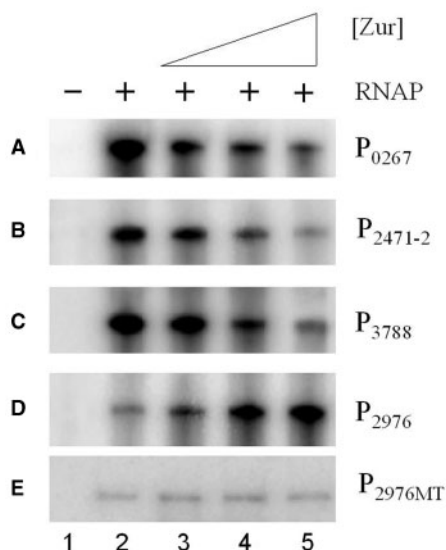


Figure 3. (A–E) Zur activates the transcription of the wild-type *XC2976* promoter (P_{2976}) but not the mutant *XC2976* promoter (P_{2976MT}) and represses *XC0267* (P_{0267}), *XC2471-2* (P_{2471-2}) and *XC3788* (P_{3788}) *in vitro*. RNA was generated *in vitro* from a PCR product template containing the promoter as well as 150-bp downstream of the start codon of the corresponding gene. For *in vitro* transcription, templates were incubated with 0 (lane 2), 7 (lane 3), 36 (lane 4) and 180 ng (lane 5) of Zur protein before the start of transcription on addition of 1 U of RNAP. A negative control (lane 1) was set up without addition of RNAP. Transcription products were then run on a 5% denatured polyacrylamide gel containing 7M urea in $1\times$ Tris borate–EDTA electrophoresis buffer. Each experiment was repeated three times and similar results were obtained.

promoter region were surveyed based on the known features of *E. coli* promoter sequence (TTGACA [-35]-N17 or N16 [spacer]-TATAAT[-10]-N6 or N7 [spacer] - +1 [transcriptional start site]) (Figure 1). Consistent with the fact that *E. coli* Zur represses gene expression by binding to the -35 to -10 region of its target promoters to block the entry of RNA polymerase (14,15), the binding site of *Xcc* Zur in all of the three tested Zur-repressed promoters overlaps the -35 to -10 region (Figure 1). Interestingly, the Zur-binding site in the Zur-activated promoter (P_{XC2976} of *XC2976*) also overlaps the -35 to -10 region (Figure 1).

The 20-bp inverted repeat sequence is essential for Zur binding and for activation of *XC2976* transcription by Zur

To determine whether the 20-bp imperfect inverted repeat in the *XC2976* promoter is indeed a Zur-binding site and

the binding of Zur with this element is essential for the transcriptional activation of *XC2976*, the left half of the 20-bp inverted repeat, CGTGATGTGA, was mutagenized and altered into ACACCACACC (see Materials and methods section for details). This mutagenized *XC2976* promoter fragment was designated as P_{2976MT} and used for construction of a *gusA* transcriptional fusion reporter plasmid named $pG2976MT$ ($P_{2976MT-gusA}$) (Figure 4A) and for EMSA with *Xcc* His₆-tagged Zur protein. As shown in Figure 4B, the His₆-tagged Zur bound specifically to the wild-type *XC2976* promoter fragment (P_{2976}) but failed to bind to the mutated *XC2976* promoter fragment P_{2976MT} in the same conditions although 20-fold of Zur protein was used, suggesting that the 20-bp imperfect inverted repeat element in the *XC2976* promoter is a critical motif for the interaction between Zur and *XC2976* promoter. Furthermore, under the *Xcc* wild-type strain 8004 background (in which the Zur protein is a wild-type), the GUS activity produced by the wild-type *XC2976* promoter-*gusA* fusion reporter plasmid $pG2976$ was 4.12 U, while the GUS activity produced by the mutagenized *XC2976* promoter-*gusA* fusion reporter plasmid $pG2976MT$ was only 0.14 U (Figure 3C). These results revealed that the Zur was unable to activate the transcription of the mutated *XC2976* promoter. To determine whether the 20-bp imperfect inverted repeat is required for *in vitro* activation of *XC2976* promoter, an *in vitro* transcription assay was performed on the mutated *XC2976* promoter in which the 20-bp imperfect inverted repeat element is lacking (see Materials and methods section for details). As expected, although activation was observed at the wild-type *XC2976* promoter (P_{2976}), no activation was detected at the mutated *XC2976* promoter (P_{2976MT}) (Figure 3C and D). Overall, these findings demonstrate that the 20-bp imperfect inverted repeat is indispensable for Zur binding and activation of *XC2976* both *in vivo* and *in vitro*.

XC2976 may be a CDF-type cation efflux pump with broad metal specificity

The above data suggest that *XC2976* may be involved in Zn^{2+} efflux. The primary function of the Zn^{2+} efflux system is to export excessive Zn^{2+} out of the cytoplasm when the intracellular Zn^{2+} exceeds a critical level. Disruption of the Zn^{2+} efflux system will lead to intracellular Zn^{2+} extreme accumulation that then affects the growth of the bacteria. To verify further if *XC2976* encodes a component of the Zn^{2+} efflux system,

at 28°C for 15 min, and analyzed by 4% polyacrylamide gel electrophoresis. (B) Effect of EDTA, Zn^{2+} and Cu^{2+} on the binding of Zur to its target promoters. Zur -, no Zur protein was added to the binding mixture; Zur +, Zur protein was added to the binding mixture to a final concentrations of 254 nM; EDTA -, no EDTA was added to the binding mixture; EDTA +, EDTA was added to the binding mixture to a final concentrations of 2 mM; Zn^{2+} -, no $ZnSO_4$ was added to the binding mixture; Zn^{2+} +, $ZnSO_4$ was added to the binding mixture to a final concentrations of 0.25 mM; Cu^{2+} -, no $CuSO_4$ was added to the binding mixture; Cu^{2+} +, $CuSO_4$ was added to the binding mixture to a final concentrations of 3 mM. (C) DNaseI footprinting analysis of Zur binding to P_{0267} , P_{2471-2} , P_{2976} and P_{3788} . Zur protein was incubated with a 5'-TAMRA-labeled DNA fragment in binding buffer at 28°C for 15 min and then DNaseI (Promega) was added to the reaction mixture and incubated for 1 min. The DNaseI digestion products were separated in 4.75% Long Ranger Gel on an ABI 377 DNA sequencer. The sequencing product of the same fragment was included for localization of the binding site. Photos of the 'Gel Image' were directly taken from the screen of the sequencer. The concentration of Zur in each reaction was 0 (line 1), 80 (line 2) or 120 (line 3) nM. Line 4, sequencing product; line 5, sequence of the indicated region. The boxed areas indicate the additional DNaseI hypersensitive sites. (D) Multiple alignment of the sequences of the Zur-binding sites in the promoters of *XC0267*, *XC2471-2* and *XC3788*, and *E. coli* Zur-box. Black boxes denote identical amino acid residues and gray-shaded boxes indicate the place where at least two of the four residues are identical.

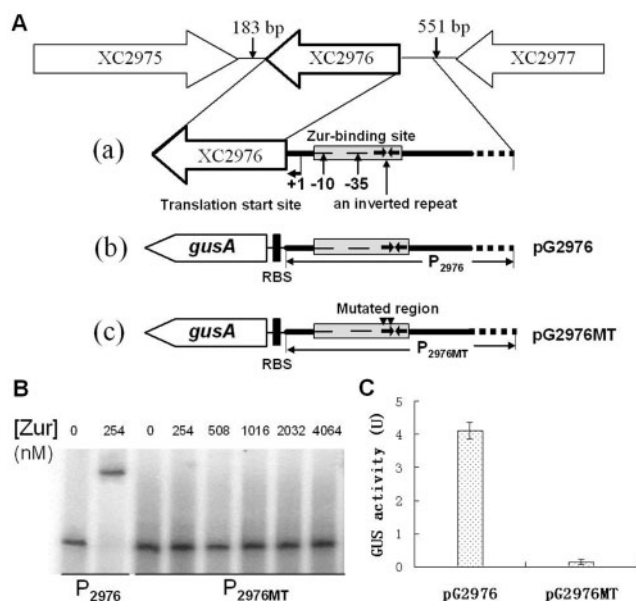


Figure 4. Effect of mutation in Zur-binding site on Zur binding and Zur transcriptional activation. (A) The detailed promoter composition of *XC2976* (a) and the genetic structure of transcriptional *gus* fusion with the wild-type (P_{2976} -*gusA*, pG2976) (b) or mutated Zur-binding site (P_{2976MT} -*gusA*, pG2976MT) (c). The identified transcriptional start site (+1), the deduced -35 and -10 promoter regions and the Zur-binding site in the *XC2976* promoter, as well as the inverted repeat inside the Zur-binding site and the altered region in the Zur-binding site are shown. (B) EMSA of Zur binding to DNA fragments with the wild-type (P_{2976}) or mutated Zur-binding site (P_{2976MT}). Zur protein was incubated with a 32 P-end-labeled DNA fragment in binding buffer at 28°C for 20 min, and analyzed by 4% polyacrylamide gel electrophoresis. (C) The GUS activity of reporter plasmids (pG2976, containing a transcriptional *gus* fusion with the wild-type *XC2976* promoter, and pG2976MT, containing a transcriptional *gus* fusion with the mutated *XC2976* promoter) in the wild-type *Xcc* strain 8004. *Xcc* strains wt/pG2976 and wt/pG2976MT were grown in NYG medium for 24 h and the GUS activity was measured as described by Jefferson *et al.* (27). Data are the mean \pm SD of triplicate measurements. Each experiment was repeated three times and similar results were obtained.

we constructed an *XC2976* mutant designated 2976nk (Table 1) by homologous suicide plasmid integration (30) and measured its intracellular Zn^{2+} content as well as its growth rate in an excessive Zn^{2+} medium (see Materials and methods section for details). The results showed that the Zn^{2+} content in the mutant cells was about four times higher than that in the wild-type cells when grown in NYG supplemented with 300 μ M of $ZnSO_4$ for 4 h (Table 4). The complemented mutant strain contained similar amount of Zn^{2+} as the wild-type (Table 4), representing that the Zn^{2+} content in the mutant cells could be restored to the wild-type level by *XC2976* *in trans*. The growth rate of the bacteria was tested in NYG medium supplemented with serial amounts of Zn^{2+} . The results showed that the mutant 2976nk grew poorly in NYG medium supplemented with 400 μ M $ZnSO_4$, whereas the wild-type strain 8004 grew essentially normally in the same conditions (Figure 5). The growth of the mutant in the same conditions could be restored to the wild-type level by *XC2976* *in trans* (Figure 5).

Table 4. The intracellular zinc content of *Xcc* strains^a

Strains	Intracellular zinc content (μ g/ 10^{10} cells)
8004 (wild-type)	1.4 \pm 0.11A
1430nk (<i>zur</i> mutant)	6.2 \pm 0.21B
2976nk (<i>XC2976</i> mutant)	6.4 \pm 0.16B
C2976nk (complemented <i>XC2976</i> mutant)	1.5 \pm 0.08A

^aData are the mean \pm SD of triplicate measurements. Each experiment was repeated three times and similar results were obtained. The different letters in each data column indicate significant differences at $P = 0.01$.

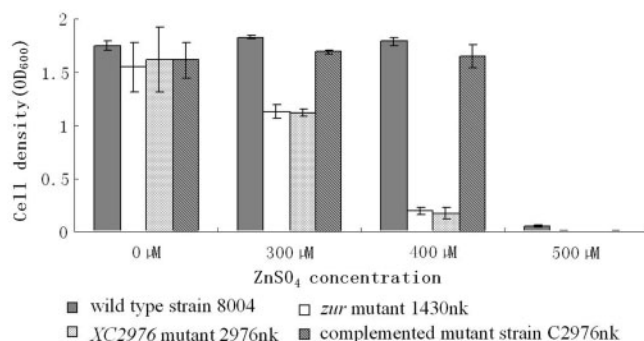


Figure 5. *XC2976* is required for *Xcc* zinc resistance and the *XC2976* and *zur* mutants display a similar zinc sensitivity level. Two hundred microliters of overnight culture (cell concentration adjusted to $OD_{600} = 1.0$) of each *Xcc* strain were inoculated into 200-ml NYG medium supplemented with $ZnSO_4$ to a final concentration of 300, 400 or 500 μ M. Cells were incubated at 28°C with shaking at 200 r.p.m. for 24 h. The cell density was measured spectrometrically at 600 nm. Values are the mean \pm SD of triplicate measurements. Each experiment was repeated three times and similar results were obtained.

These results demonstrate that *XC2976* may encode a protein involved in Zn^{2+} efflux system of *Xcc*.

As shown in Table 2, *XC2976* displays 67% similarity at the amino acid level to CzcD, a CDF (cation-diffusion facilitators)-type heavy metal ion efflux system of *Ralstonia metallidurans* (33). CDFs consist of a protein family involved in the metal ion transport found in archaea, eubacteria and eukarya (36). *Ralstonia metallidurans* CzcD is a membrane-bound protein with six transmembrane (TM) regions and contributes to the Zn^{2+} resistance of the bacterium through reducing the intracellular accumulation of the cations (33,37). To test whether *XC2976* possesses any structural similarity to *R. metallidurans* CzcD, we performed a TM domain analysis of *XC2976* using the DAS TM prediction server (<http://www.biomed.su.se/~server/DAS>) (38). The TM-segment prediction result showed that, like CzcD, *XC2976* is also a membrane-bound protein with six TM regions (Data not shown), suggesting further that *XC2976* may be a CDF-type Zn^{2+} efflux pump.

In addition to the effect on Zn^{2+} , the *R. metallidurans* CzcD also contributes to Co^{2+} and Cd^{2+} resistance of the bacterium. To assess whether the *czcD* homologous *XC2976* is responsible for the resistance of *Xcc* to cobalt and cadmium, the sensitivity of the *XC2976*-mutant

2976nk to cobalt and cadmium was tested by measuring its growth rate in NYG medium supplemented with serial amounts of Co^{2+} or Cd^{2+} . The results showed that the mutant failed to grow in NYG medium supplemented with 250 μM CoCl_2 or 60 μM CdSO_4 , whereas the wild-type strain grew almost normally under the same conditions (data not shown). The Co^{2+} and Cd^{2+} sensitivity of the mutant could be restored to the wild-type level by introduction of *XC2976* *in trans* (data not shown). We further found that the mutant 2976nk is more sensitive to Ni^{2+} than the wild-type (data not shown). These demonstrate that *XC2976* is required for the resistance of *Xcc* not only to Zn^{2+} but also to Co^{2+} , Cd^{2+} and Ni^{2+} , implying that *XC2976* may be a metal efflux pump with broad substrate spectrum.

The above results demonstrate that the Zur of *Xcc* represses Zn^{2+} uptake and activates Zn^{2+} export. In Zn^{2+} -rich medium, the *zur* mutant accumulated significantly more Zn^{2+} than the wild-type (11). To determine whether the increased Zn^{2+} accumulation in the *zur* mutant cells is due to the disturbance of the effusion or over-uptake of Zn^{2+} , or both, we compared the Zn^{2+} sensitivity and the intracellular Zn^{2+} accumulation of the *zur* mutant 1430nk and the *XC2976*-mutant 2976nk. The results showed that the Zn^{2+} sensitivity levels and the intracellular Zn^{2+} accumulation of the *zur* mutant are identical to that of the *XC2976*-mutant (Figure 5; Table 4), indicating that the increased intracellular Zn^{2+} accumulation is due to the deficiency of Zn^{2+} effusion. This suggests that the Zn^{2+} hypersensitivity of the *zur* mutant is attributed to the suppression of *XC2976* expression, not the constitutive expression of Zn^{2+} -uptake systems.

DISCUSSION

Our previous observation that the *Xcc zur*-mutant accumulates significantly more Zn^{2+} than the parent strain suggests that Zur may regulate the zinc uptake and/or export systems in *Xcc* (11). By DNA microarray hybridization, sequence homology comparison, mutagenesis and promoter-reporter analysis, here we have identified three genes coding for putative zinc uptake systems and one gene encoding a zinc export system, which are regulated by Zur in *Xcc*. Of which, *XC2976* encodes a Zn^{2+} efflux pump with broad metal substrate spectrum and is positively regulated by Zur, and *XC0267*, *XC2471* as well as *XC3788* encode putative Zn^{2+} -uptake systems and are negatively regulated by Zur. DNA microarray hybridization result displayed that the expression of more than 60 ORFs was affected by Zur in *Xcc*. For our interests in this study, we focused on the above four zinc-homeostasis genes and investigated the mechanisms by which Zur regulates their expression.

The results present in this article reveal that the expression of the Zn^{2+} export gene *XC2976* and the putative Zn^{2+} -uptake genes *XC0267*, *XC2471-2* and *XC3788* responses to Zn^{2+} through the mediation of Zur, and Zur binds to the promoters of these genes in a Zn^{2+} -dependent manner. Such influence on the expression

of Zn^{2+} -uptake systems has been observed in other bacteria (3,4). DNaseI footprinting analyses showed that the Zur-binding sequence in the three Zur-repressing promoters is an about 30-bp AT-rich sequence, which overlaps the promoters' -35 to -10 region. This is similar to the findings from previous studies on *E. coli* and other bacteria. *E. coli* Zur represses the transcription of the Zn^{2+} -uptake operon *znuABC* by binding to a ~30-bp AT-rich sequence overlapping the -35 to -10 region of the *znuABC* promoter to block the entry of the RNA polymerase (14,15). The Zur of *B. subtilis* (16), *M. tuberculosis* (12) and *S. coelicolor* (17) also binds to a 30-bp AT-rich sequence overlapping the -35 to -10 region of its target promoters. These may suggest that most bacterial Zurs including the *Xcc* Zur, if not all, regulate negatively the expression of Zn^{2+} -uptake systems by a mechanism similar to that of *E. coli* Zur.

To our knowledge, this is the first observation that a bacterial Zur positively regulates a Zn^{2+} -export system besides being a repressor for Zn^{2+} -uptake systems. It has been generally considered that the Fur family regulators function as repressors and bacterial metal ion uptake and efflux systems are separately regulated by their own regulators, i.e. members of the MerR and ArsR/SmtB family transcriptional regulators control metal ion efflux systems, while members of the Fur family transcriptional regulators control metal ion uptake systems (3,4,39). In *E. coli*, for example, the expression of *zntA*, a gene encoding a P-type ATPase- Zn^{2+} -export system, is regulated by the MerR family transcriptional regulator ZntR (40), while the expression of the high-affinity Zn^{2+} -uptake system *znuABC* is regulated by the Fur family transcriptional regulator Zur (5). A homology survey displayed that the genome of the *Xcc* strain 8004 possesses a ZntA [XC3531 (GenBank accession number YP_244594)] and two ArsR/SmtB [XC1489 (GenBank accession number YP_242577) and XC2723 (GenBank accession number YP_243792)] homologues but not ZntR homologue. These facts, taken together, suggest that *Xcc* may have developed its specific zinc homeostasis machinery, the regulatory mechanism of which is distinct from that of *E. coli*. A few members of the Fur family have been found to exhibit repressor and activator functions. For instance, the Fur of *Neisseria meningitidis* (41) and *Vibrio vulnificus* (42), the BosR of *Borrelia burgdorferi* (43) and the PerR of *B. subtilis* (44) have been demonstrated to act as an activator and a repressor. These regulators suppress genes' transcription by binding to their target promoter regions and activate genes' transcription by binding to an upstream sequence of promoters (41-44). In addition to negative regulation of Zn^{2+} -uptake systems, a few Zur proteins have been found to affect positively the expression of genes with cellular functions beyond the metal ion homeostasis. For example, the Zur of *B. subtilis* positively regulates the expression of the genes *rocD* and *rocE* that are involved in amino acid transport (16), and the Zur of *S. typhimurium* activates the expression of the virulence-related operon *fliAZ* (9). Although it has been proposed that the *B. subtilis* Zur may activate the expression of *rocE* and *rocD* indirectly (16), the molecular mechanism by which Zur positively

regulates gene expression in these bacteria is unknown. The DNA microarray hybridization showed that in addition to the four zinc homeostasis-related genes, the *Xcc* Zur also regulates other more than 60 genes involved in other cellular processes including *hpaB* and *hrpE*, which are involved in pathogenicity and hypersensitive response (Table S2). It is a subject of considerable interest to investigate the mechanisms by which Zur regulates these processes in *Xcc*.

The *gus*-promoter transcriptional fusion analysis suggests that the expression of *XC2976* responses to Zn^{2+} through Zur mediation. The EMSA and DNaseI footprinting analyses demonstrate that the *Xcc* Zur binds to the promoter region of the Zn^{2+} -export gene *XC2976*. *In vitro* transcription assay demonstrates that Zur activates the transcription of *XC2976* directly. These results reveal that the *Xcc* Zur directly activates the expression of *XC2976*. Interestingly, the *Xcc* Zur binds to a 59-bp GC-rich sequence with a 20-bp imperfect inverted repeat overlapping the -35 to -10 region of the *XC2976* promoter, which differs from the ~30-bp AT-rich sequence bound by Zur in the promoters of the Zn^{2+} -uptake systems. Mutagenesis of the 20-bp imperfect inverted repeat resulted in complete abolishment of the *in vitro* binding of Zur to the promoter, and the *in vivo* and *in vitro* activation of the *XC2976* promoter by Zur, indicating that the *Xcc* Zur positively regulates the transcription of *XC2976* by directly binding to a *cis*-acting element located in the *XC2976* promoter. How the *Xcc* Zur distinguishes these two different target sequences and what is the mechanism by which it activates the transcription of the Zn^{2+} -export gene *XC2976* after binding to the promoter remain to be further investigated.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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REFERENCES

- Nies, D.H. (1999) Microbial heavy-metal resistance. *Appl. Microbiol. Biotechnol.*, **51**, 730–750.
- Blencowe, D.K. and Morby, A.P. (2003) Zn(II) metabolism in prokaryotes. *FEMS Microbiol. Rev.*, **27**, 291–311.
- Hantke, K. (2001) Bacterial zinc transporters and regulators. *Biomol.*, **14**, 239–249.
- Hantke, K. (2005) Bacterial zinc uptake and regulators. *Curr. Opin. Microbiol.*, **8**, 196–202.
- Patzer, S.I. and Hantke, K. (1998) The ZnuABC high-affinity zinc uptake system and its regulator Zur in *Escherichia coli*. *Mol. Microbiol.*, **28**, 1199–1210.
- Gaballa, A. and Helmann, J.D. (1998) Identification of a zinc-specific metalloregulatory protein, Zur, controlling zinc transport operons in *Bacillus subtilis*. *J. Bacteriol.*, **180**, 5815–5821.
- Dalet, K., Gouin, E., Cenatiempo, Y., Cossart, P. and Hechard, Y. (1999) Characterisation of a new operon encoding a Zur-like protein and an associated ABC zinc permease in *Listeria monocytogenes*. *FEMS Microbiol. Lett.*, **174**, 111–116.
- Lindsay, J.A. and Foster, S.J. (2001) *zur*: a Zn^{2+} -responsive regulatory element of *Staphylococcus aureus*. *Microbiology.*, **147**, 1259–1266.
- Campoy, S., Jara, M., Busquets, N., Perez De Rozas, A.M., Badiola, I. and Barbe, J. (2002) Role of the high-affinity zinc uptake *znuABC* system in *Salmonella enterica* serovar typhimurium virulence. *Infect. Immun.*, **70**, 4721–4725.
- Garrido, M.E., Bosch, M., Medina, R., Llagostera, M., Perez de Rozas, A.M., Badiola, I. and Barbe, J. (2003) The high-affinity zinc-uptake system *znuACB* is under control of the iron-uptake regulator (*fur*) gene in the animal pathogen *Pasteurella multocida*. *FEMS Microbiol. Lett.*, **221**, 31–37.
- Tang, D.-J., Li, X.-J., He, Y.-Q., Feng, J.-X., Chen, B. and Tang, J.-L. (2005) The zinc uptake regulator *Zur* is essential for the full virulence of *Xanthomonas campestris* pv. *campestris*. *Mol. Plant Microbe Interact.*, **18**, 652–658.
- Maciag, A., Dainese, E., Rodriguez, G.M., Milano, A., Proveddi, R., Pasca, M.R., Smith, I., Palu, G., Riccardi, G. and Manganeli, R. (2007) Global analysis of *Mycobacterium tuberculosis* Zur (FurB) regulon. *J. Bacteriol.*, **189**, 730–740.
- Shin, J.H., Oh, S.Y., Kim, S.J. and Roe, J.H. (2007) The zinc-responsive regulator *Zur* controls a zinc uptake system and some ribosomal proteins in *Streptomyces coelicolor* A3(2). *J. Bacteriol.*, **189**, 4070–4077.
- Patzer, S.I. and Hantke, K. (2000) The zinc-responsive regulator *Zur* and its control of the *znu* gene cluster encoding the ZnuABC zinc uptake system in *Escherichia coli*. *J. Biol. Chem.*, **275**, 24321–24332.
- Oутten, C.E. and O'Halloran, T.V. (2001) Femtomolar sensitivity of metalloregulatory proteins controlling zinc homeostasis. *Science.*, **292**, 2488–2492.
- Gaballa, A., Wang, T., Ye, R.W. and Helmann, J.D. (2002) Functional analysis of the *Bacillus subtilis* Zur regulon. *J. Bacteriol.*, **184**, 6508–6514.
- Owen, G.A., Pascoe, B., Kallifidas, D. and Paget, M.S. (2007) Zinc-responsive regulation of alternative ribosomal protein genes in *Streptomyces coelicolor* involves *zur* and *sigmaR*. *J. Bacteriol.*, **189**, 4078–4086.
- Hayward, A.C. (1993) In Swings, J.G. and Civerolo, E.L. (eds), *Xanthomonas*, Chapman & Hall, London, pp. 51–54.
- Miller, J.H. (1972) *Experiments in Molecular Genetics*. New York, Cold Spring Harbour Laboratory Press.
- Daniels, M.J., Barber, C.E., Turner, P.C., Sawczyk, M.K., Byrde, R.J.W. and Fielding, A.H. (1984) Cloning of genes involved in pathogenicity of *Xanthomonas campestris* pv. *campestris* using the broad-host-range cosmid pLAFR1. *EMBO J.*, **3**, 3323–3328.
- Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Turner, P., Barber, C.E. and Daniels, M.J. (1985) Evidence for clustered pathogenicity genes in *Xanthomonas campestris* pv. *campestris*. *Mol. Gen. Genet.*, **199**, 338–343.
- Qian, W., Jia, Y., Ren, S.-X., He, Y.-Q., Feng, J.-X., Lu, L.-F., Sun, Q., Ying, G., Tang, D.-J., Tang, H. *et al.* (2005) Comparative and functional genomic analyses of the pathogenicity of phytopathogen *Xanthomonas campestris* pv. *campestris*. *Genome Res.*, **15**, 757–767.
- He, Y.-Q., Zhang, L., Jiang, B.-L., Zhang, Z.-C., Xu, R.-Q., Tang, D.-J., Qin, J., Jiang, W., Zhang, X., Liao, J. *et al.* (2007) Comparative and functional genomics reveals genetic diversity and determinants of host specificity among reference strains and a large collection of Chinese isolates of the phytopathogen *Xanthomonas campestris* pv. *campestris*. *Genome Biol.*, **8**, R218.
- Yang, H., Haddad, H., Tomas, C., Alsaker, K. and Papoutsakis, E.T. (2003) A segmental nearest neighbor normalization and gene

- identification method gives superior results for DNA-array analysis. *Proc. Natl Acad. Sci. USA.*, **100**, 1122–1127.
26. Kuwayama, H., Obara, S., Morio, T., Katoh, M., Urushihara, H. and Tanaka, Y. (2002) PCR-mediated generation of a gene disruption construct without the use of DNA ligase and plasmid vectors. *Nucleic Acids Res.*, **30**, E2.
 27. Jefferson, R.A., Burges, S.M. and Hirsh, D. (1986) β -glucuronidase from *Escherichia coli* as a gene-fusion marker. *Proc. Natl Acad. Sci. USA.*, **83**, 8447–8451.
 28. Merighi, M., Majerczak, D.R., Zianni, M., Tessanne, K. and Coplin, D.L. (2006) Molecular characterization of *Pantoea stewartii* subsp. *stewartii* HrpY, a conserved response regulator of the Hrp type III secretion system, and its interaction with the *hrpS* promoter. *J. Bacteriol.*, **188**, 5089–5100.
 29. Friedman, Y.E. and O'Brian, M.R. (2004) The ferric uptake regulator (Fur) protein from *Bradyrhizobium japonicum* is an iron-responsive transcriptional repressor *in vitro*. *J. Biol. Chem.*, **279**, 32100–32105.
 30. Windgassen, M., Urban, A. and Jaeger, K.E. (2000) Rapid gene inactivation in *Pseudomonas aeruginosa*. *FEMS Microbiol. Lett.*, **193**, 201–205.
 31. Schafer, A., Tauch, A., Jager, W., Kalinowski, J., Thierbach, G. and Puhler, A. (1994) Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. *Gene.*, **145**, 69–73.
 32. Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene.*, **33**, 103–119.
 33. Anton, A., Grosse, C., Reissmann, J., Pribyl, T. and Nies, D.H. (1999) CzcD is a heavy metal ion transporter involved in regulation of heavy metal resistance in *Ralstonia* sp. strain CH34. *J. Bacteriol.*, **181**, 6876–6881.
 34. Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) Basic local alignment search tool. *J. Mol. Biol.*, **215**, 403–410.
 35. Zang, N., Tang, D.-J., Wei, M.-L., He, Y.-Q., Chen, B., Feng, J.-X., Xu, J., Gan, Y.-Q., Jiang, B.-L. and Tang, J.-L. (2006) Requirement of a mip-like gene for virulence in the phytopathogenic bacterium *Xanthomonas campestris* pv. *campestris*. *Mol. Plant Microbe Interact.*, **20**, 21–30.
 36. Nies, D.H. (2003) Efflux-mediated heavy metal resistance in prokaryotes. *FEMS Microbiol. Rev.*, **27**, 313–339.
 37. Munkelt, D., Grass, G. and Nies, D.H. (2004) The chromosomally encoded cation diffusion facilitator proteins DmeF and FieF from *Wautersia metallidurans* CH34 are transporters of broad metal specificity. *J. Bacteriol.*, **186**, 8036–8043.
 38. Rost, B., Fariselli, P. and Casadio, R. (1996) Topology prediction for helical transmembrane proteins at 86% accuracy. *Protein Sci.*, **5**, 1704–1718.
 39. Moore, C.M. and Helmann, J.D. (2005) Metal ion homeostasis in *Bacillus subtilis*. *Curr. Opin. Microbiol.*, **8**, 188–195.
 40. Brocklehurst, K.R., Hobman, J.L., Lawley, B., Blank, L., Marshall, S.J., Brown, N.L. and Morby, A.P. (1999) ZntR is a Zn(II)-responsive MerR-like transcriptional regulator of *zntA* in *Escherichia coli*. *Mol. Microbiol.*, **31**, 893–902.
 41. Delany, I., Rappuoli, R. and Scarlato, V. (2004) Fur functions as an activator and as a repressor of putative virulence genes in *Neisseria meningitidis*. *Mol. Microbiol.*, **52**, 1081–1090.
 42. Lee, H.J., Bang, S.H., Lee, K.H. and Park, S.J. (2007) Positive regulation of *fur* gene expression via direct interaction of Fur in a pathogenic bacterium, *Vibrio vulnificus*. *J. Bacteriol.*, **189**, 2629–2636.
 43. Boylan, J.A., Posey, J.E. and Gherardini, F.C. (2003) Borrelia oxidative stress response regulator, BosR: a distinctive Zn-dependent transcriptional activator. *Proc. Natl Acad. Sci. USA.*, **100**, 11684–11689.
 44. Hayashi, K., Ohsawa, T., Kobayashi, K., Ogasawara, N. and Ogura, M. (2005) The H₂O₂ stress-responsive regulator PerR positively regulates *srfA* expression in *Bacillus subtilis*. *J. Bacteriol.*, **187**, 6659–6667.
 45. Huynh, T.V., Dahlbeck, D. and Staskawicz, B.J. (1989) Bacterial blight of soybean: regulation of a pathogen gene determining host cultivar specificity. *Science.*, **245**, 1374–1377.
 46. Leong, S.A., Ditta, G.S. and Helinski, D.R. (1982) Heme biosynthesis in *Rhizobium*. Identification of a cloned gene coding for delta-aminolevulinic acid synthetase from *Rhizobium meliloti*. *J. Biol. Chem.*, **257**, 8724–8730.