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 Zn\(^{2+}\)-uptake systems, the Zur of the bacterial phytopathogen *Xanthomonas campestris* pathovar *campestris* (*Xcc*) acts as an activator of a Zn\(^{2+}\) 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 Zn\(^{2+}\)-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 Zn\(^{2+}\)-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 Zn\(^{2+}\) level must be precisely regulated. In bacteria, the balance between Zn\(^{2+}\) 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 Zn\(^{2+}\)-sensing metalloregulatory protein belonging to the Fur (Ferric uptake regulator) regulatory protein family, to repress the transcription of Zn\(^{2+}\)-uptake systems to maintain a delicate homeostasis (3,4). Zur was first described as a negative transcriptional regulator of genes encoding Zn\(^{2+}\)-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 Zn\(^{2+}\)-uptake...
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 *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 campesstris* pathovar *campesstris* (*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

<table>
<thead>
<tr>
<th>Strains or plasmids</th>
<th>Relevant characteristics</th>
<th>Reference or source</th>
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</thead>
<tbody>
<tr>
<td>E. coli</td>
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<td></td>
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<tr>
<td>JM109</td>
<td><em>RecA1, endA1, gyrA96, thi, supE44, relA1 Δ (lac-proAB)/F’</em> [traD6, lacP, lacZ Δ M15]</td>
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<tr>
<td>BL21(DE3) pLysS</td>
<td>F’ Φ80 λDE3 (rrnB4-KAN) gal dcm (DE3) pLysS</td>
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<tr>
<td>BL1430</td>
<td>BL21(DE3)pLysS harboring pET1430, Kan’</td>
<td>This work</td>
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<tr>
<td>Xcc</td>
<td>Wild-type, Rifr</td>
<td>(20)</td>
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<tr>
<td>8004</td>
<td>As 8004, but XC1430 (zur):pK18mob, Rif’, Kan’</td>
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<tr>
<td>1430nk</td>
<td>As 8004, but XC2976:pK18mob, Rif’, Kan’</td>
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<tr>
<td>2976nk</td>
<td>2976nk harboring pXC2976, Rif’, Kan’, Te’</td>
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<td>pET-30a-C(+)</td>
<td>Expression vector, allow the production of fusion proteins containing amino terminal 6xHis-tagged sequences. Kanr</td>
<td>Novagen, Germany</td>
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<td>pXC2976</td>
<td>pLAFR6 containing a 1349-bp fragment including XC2976 gene, Te’</td>
<td>This work</td>
</tr>
</tbody>
</table>

* Rif’, Kan’, Spe’, Te’ = 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 (WT) and the mutant 1430 nk, 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, 1.4M 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™ (Eric 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 recombined 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 filtered 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) 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 filtered into a 50 ml centrifugation tube and mixed with 2.5 ml of prewashed Ni-NTA resin (Qiagen) and then washed several times with 50 ml of precooled wash buffer (50 mM NaH2PO4, 300 mM NaCl, 20 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::Tn5gusAS as template and the primer pair GusAF/GusAR (Table S1). Each of the amplified promoter fragments and the 1.8-kg 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 wild-type strain 8004 (Table S1). Firstly, a 130- and 195-bp DNA fragments were respectively amplified by PCR using the total DNA of the wild-type strain 8004 as template and the primer sets 0267PF/0267PR and 2976PF/2976PR (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).

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 P0267-F/P0267-R, P2471-2-F/P2471-2-R, P2976-F/P2976-R, P3788-F/P3788-R and IGR2471-2-F/IGR2471-2-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 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] 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 µl 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 P0267-R, P2471-2-R, P3788-R and P2976-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 ng 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 P2676-F, P2471-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 ‘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 used as template for PCR reaction. To improve assay sensitivity, two rounds of semi-nested PCR were performed using the total DNA of the wild-type strain 8004 as template and 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 XCO267, XC2471-2, XC2976 and XC3788 were performed using the experimental procedure described by Friedman et al. (29). The promoter template DNA fragments of XCO267 (P2676), 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 P2976MT, 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/2976ivrR (Table S1), and fragments A and B were then linked together to generate the P2976MT fragment by fusion PCR (26). Each promoter template fragment includes the promoter region as well as ∼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, 1 U RNase inhibitor, 100 μM ZnSO4] containing 4 mM 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) (Epitroch, 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 7 M urea in 1× Tris borate–EDTA electrophoresis buffer. The transcripts obtained were analyzed by a phosphorimag.jpger 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 E. coli strain 8004 by triparental transformation. 

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 37°C for 20 min, immediately prior to use, 5 μl 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.Columns (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.
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 \( Xcc_{2976} \) 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 \( Xcc_{2976} \). 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 \( Xcc_{2976} \) mutant 2976nk, a 1349-bp DNA fragment containing the entire \( Xcc_{2976} \) 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 triparental 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-μl 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 spectrophotometrically 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 \( \text{ZnSO}_4 \): 0, 300, 400 and 500 μM; \( \text{CoCl}_2 \): 0, 150, 200, 250, 300 and 350 μM; \( \text{CdSO}_4 \): 0, 40, 50, 60, 70 and 80 μM and \( \text{NiSO}_4 \): 0, 400, 500, 600, 700 and 800 μM.

**Measurement of \( \text{Zn}^{2+} \) 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 \( \text{ZnSO}_4 \) to a final concentration of 300 μM 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 \( \text{Zn}^{2+} \) 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 \( \text{Zn}^{2+} \)-uptake systems and positively regulates a gene related to a \( \text{Zn}^{2+} \) efflux pump

Our previous work showed that in \( Xcc \) inactivation of \( zur \) resulted in hypersensitivity to \( \text{Zn}^{2+} \) toxicity (11), indicating that certain important genes involved in \( \text{Zn}^{2+} \) 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 \( \text{Zn}^{2+} \)-replete medium NYG (ca. 10 μM \( Zn^{2+} \)) (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 \( Xcc_{2976} \), which was predicted to encode a cobalt–zinc–cadmium resistant protein (23) showing 67% amino acid sequence similarity to the \( \text{Zn}^{2+} \) efflux pump CzD 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 \( Xcc_{2976} \), the predicted products encoded by \( XCC_{2471} \) and \( XCC_{3788} \) displayed amino acid sequence similarity to the \( \text{Zn}^{2+} \) homeostasis-related proteins identified in other bacteria. \( XCC_{2471} \) and \( XCC_{2067} \) showed 29 and 54% amino acid sequence similarities to \( YciA \) and \( YciC \) of *B. subtilis*, respectively, while \( XCC_{3788} \) exhibited 38% similarity to \( ZnuC \) of *E. coli* (Table 2). \( YciA \) and \( YciC \) are components of the low-affinity \( \text{Zn}^{2+} \)-uptake system YciABC of *B. subtilis* (16) and \( ZnuC \) is a member of the high-affinity \( \text{Zn}^{2+} \)-uptake system \( ZnuABC \) of *E. coli* (5). Based on these similarities, we propose that \( XCC_{2976} \) is a putative \( \text{Zn}^{2+} \) efflux-related gene and \( XCC_{2067} \), \( XCC_{2471} \) as well as \( XCC_{3788} \) are putative \( \text{Zn}^{2+} \) 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 \( \text{Zn}^{2+} \) homeostasis-related genes \( XCC_{2067} \), \( XCC_{2471} \), \( XCC_{3788} \) and \( XCC_{2976} \) in the genome of \( Xcc \) strain 8004 are shown in Table 2. \( XCC_{2067} \) and its adjacent ORF \( XCC_{2066} \) are separated by a spacer with only 61 bp and their transcriptions are back to back (Figure 1). The ORFs \( XCC_{2471} \) and \( XCC_{2472} \) 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
The expression of XC0267, XC2471-2, XC2976 and XC3788 responses to Zn\(^{2+}\) through the mediation of Zur

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

The results showed that when the Zn\(^{2+}\) chelator EGTA was added into the Zn\(^{2+}\)-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 Zn\(^{2+}\) in the medium, because addition of ZnSO\(_4\) 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 Zn\(^{2+}\)-rich NYG medium (Table 3). The results demonstrate that the expression of XC0267, XC2471-2 and XC3788 are repressed in Zn\(^{2+}\)-rich condition and induced in Zn\(^{2+}\)-limited condition. On the contrary, XC2976 is induced in Zn\(^{2+}\)-rich condition and repressed in Zn\(^{2+}\)-limited condition. These further support the possibility that XC0267, XC2471-2 and XC3788 may play roles in Zn\(^{2+}\) uptake-related function and XC2976 in Zn\(^{2+}\) 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 and zurmt/pG3788 in NYG medium were not affected by the Zn\(^{2+}\) status in the NYG medium (Table 3). These results suggest that the Zn\(^{2+}\) 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.
showed that addition of Zur to the in vitro reactions decreased the transcription from the promoters of XCO267, XCO2471-2 and XCO3788, but increased the transcription from the XCO2976 promoter (Figure 3A–D). These demonstrate that Zur represses the transcription of XCO267, XCO2471-2 and XCO3788, and activates the transcription of XCO2976, 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 Histagged Zur protein bound to a region of 59-bp in length in the promoter of XCO2976 and an about 30-bp region in the promoters of XCO267, XCO2471-2 and XCO3788. Sequencing analysis displayed that the Zur protected regions in the promoters of XCO267, XCO2471-2 and XCO3788 are about 30-bp AT-rich sequences showing high similarity to the E. coli Zur-binding sequences (Zur-box) (AAGTGTGATATTATAACATTTCATGACTA) (14,15) (Figure 2D). Multiple alignment of the Zur-protected sequences in the promoters of XCO267, XCO2471-2 and XCO3788 as well as the E. coli Zur box using the Vector NTI Alignment program (Invitrogen) revealed a conserved core sequence aagTGTg(i)ATAaagTaaCATTt (Figure 2D). Interestingly, the Zur-protected region in the XCO2976 promoter is a 59-bp GC-rich (57.7%) sequence (AATCCCGG TGCGCCAACGCCTCTGATAATGTGGCCGCGCAC) (14,15) (Figure 2D). Multi-

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

<table>
<thead>
<tr>
<th>Reporter strain</th>
<th>GUS activity (U)</th>
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<tbody>
<tr>
<td>wt/pG0267</td>
<td>0.12 ± 0.02A</td>
</tr>
<tr>
<td>zurmt/pG0267</td>
<td>1.78 ± 0.01C</td>
</tr>
<tr>
<td>wt/pG2471-2</td>
<td>0.04 ± 0.01A</td>
</tr>
<tr>
<td>zurmt/pG2471-2</td>
<td>0.21 ± 0.03C</td>
</tr>
<tr>
<td>wt/pG2978</td>
<td>0.13 ± 0.01A</td>
</tr>
<tr>
<td>zurmt/pG3788</td>
<td>4.04 ± 0.34C</td>
</tr>
<tr>
<td>wt/pG2976</td>
<td>4.46 ± 0.10A</td>
</tr>
<tr>
<td>zurmt/pG2976</td>
<td>0.49 ± 0.01C</td>
</tr>
</tbody>
</table>

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 Histagged 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 XCO267, XCO2471-2, XCO2976 and XCO3788 were amplified by PCR using the total DNA of the Xcc wild-type strain 8004 as template and the primer sets P0267-F/P0267-R, P2471-2-F/P2471-2-R, P2976-F/P2976-R and P3788-F/P3788-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 for details). 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 XCO2471 and XCO2472 (IGR2471-2472) (Figure 2A). The purified Histagged 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 XCO267, XCO2471-2 and XCO3788.

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 XCO2976 and represses XCO267, XCO2471-2 and XCO3788, in vitro transcription assays were performed on the promoter DNA of these genes (see Materials and methods section for details). The results
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_{XC0267}), XC2471-2 (P_{XC2471-2}), XC2976 (P_{XC2976}) and XC3788 (P_{XC3788}). Zur protein was incubated with 3 pmol of 32P-end-labeled DNA fragment in binding buffer.
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 P2976MT and used for construction of a gusA transcriptional fusion reporter plasmid named pG2976MT (P2976MT-gusA) (Figure 4A) and for EMSA with Xcc His6-tagged Zur protein. As shown in Figure 4B, the His6-Zur bound specifically to the wild-type XC2976 promoter fragment (P2976) but failed to bind to the mutated XC2976 promoter fragment P2976MT 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 (P2976), no activation was detected at the mutated XC2976 promoter (P2976MT) (Figure 3C and D). Overall, these findings demonstrate that the 20-bp imperfect inverted repeat 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, the 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 (PXc2976 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
we constructed an *Xc*2976 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 $\mu$M 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 was restored to the wild-type level by *Xc*2976 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 $\mu$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 *Xc*2976 in trans (Figure 5).

### Table 4. The intracellular zinc content of *Xc* strains$^a$

<table>
<thead>
<tr>
<th>Strains</th>
<th>Intracellular zinc content ($\mu$g/10$^{10}$ cells)</th>
</tr>
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<tbody>
<tr>
<td>8004 (wild-type)</td>
<td>1.4 ± 0.11A</td>
</tr>
<tr>
<td>1430nk (<em>zur</em> mutant)</td>
<td>6.2 ± 0.21B</td>
</tr>
<tr>
<td>2976nk (<em>Xc</em>2976 mutant)</td>
<td>6.4 ± 0.16B</td>
</tr>
<tr>
<td>C2976nk (complemented <em>Xc</em>2976 mutant)</td>
<td>1.5 ± 0.08A</td>
</tr>
</tbody>
</table>

$^a$Data are the mean ± 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 $P = 0.01$.

These results demonstrate that *Xc*2976 may encode a protein involved in Zn$^{2+}$ efflux system of *Xc*.

As shown in Table 2, *Xc*2976 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 *Xc*2976 possesses any structural similarity to *R. metallidurans* CzcD, we performed a TM domain analysis of *Xc*2976 using the DAS TM prediction server (http://www.biomi.di.su.se/-server/DAS) (38). The TM-segment prediction result showed that, like CzcD, *Xc*2976 is also a membrane-bound protein with six TM regions (Data not shown), suggesting further that *Xc*2976 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 *Xc*2976 is responsible for the resistance of *Xc* to cobalt and cadmium, the sensitivity of the *Xc*2976-mutant
2976nk to cobalt and cadmium was tested by measuring its growth rate in NYG medium supplemented with serial amounts of Co\textsuperscript{2+} or Cd\textsuperscript{2+}. The results showed that the mutant failed to grow in NYG medium supplemented with 250\,\mu M CoCl\textsubscript{2} or 60\,\mu M CdSO\textsubscript{4}, whereas the wild-type strain grew almost normally under the same conditions (data not shown). The Co\textsuperscript{2+} and Cd\textsuperscript{2+} sensitivity of the mutant could be restored to the wild-type level by introduction of \textit{Xc}2976 \textit{in trans} (data not shown). We further found that the mutant 2976nk is more sensitive to Ni\textsuperscript{2+} than the wild-type (data not shown). These demonstrate that \textit{Xc}2976 is required for the resistance of \textit{Xc} not only to Zn\textsuperscript{2+} but also to Co\textsuperscript{2+}, Cd\textsuperscript{2+} and Ni\textsuperscript{2+}, implying that \textit{Xc}2976 may be a metal efflux pump with broad substrate spectrum.

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

**DISCUSSION**

Our previous observation that the \textit{Xc} \textit{zur}-mutant accumulates significantly more Zn\textsuperscript{2+} than the parent strain suggests that Zur may regulate the zinc uptake and/or export systems in \textit{Xc} (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 \textit{Xc}. Of which, \textit{Xc}2976 encodes a Zn\textsuperscript{2+} efflux pump with broad metal substrate spectrum and is positively regulated by Zur, and \textit{Xc}0267, \textit{Xc}2471 as well as \textit{Xc}3788 encode putative Zn\textsuperscript{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 \textit{Xc}. 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\textsuperscript{2+} export gene \textit{Xc}2976 and the putative Zn\textsuperscript{2+}-uptake genes \textit{Xc}0267, \textit{Xc}2471-2 and \textit{Xc}3788 responses to Zn\textsuperscript{2+} through the mediation of Zur, and Zur binds to the promoters of these genes in a Zn\textsuperscript{2+}-dependent manner. Such influence on the expression of Zn\textsuperscript{2+}-uptake systems has been observed in other bacteria (3,4). DNaseI footprint 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 \textit{E. coli} and other bacteria. \textit{E. coli} Zur represses the transcription of the Zn\textsuperscript{2+}-uptake operon \textit{znuABC} by binding to a 30-bp AT-rich sequence overlapping the −35 to −10 region of the \textit{znuABC} promoter to block the entry of the RNA polymerase (14,15). The Zur of \textit{B. subtilis} (16), \textit{M. tuberculosis} (12) and \textit{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 \textit{Xc} Zur, if not all, regulate negatively the expression of Zn\textsuperscript{2+}-uptake systems by a mechanism similar to that of \textit{E. coli} Zur.

To our knowledge, this is the first observation that a bacterial Zur positively regulates a Zn\textsuperscript{2+}-export system besides being a repressor for Zn\textsuperscript{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 \textit{E. coli}, for example, the expression of \textit{zntA}, a gene encoding a P-type ATPase-Zn\textsuperscript{2+}-export system, is regulated by the MerR family transcriptional regulator ZntR (40), while the expression of the high-affinity Zn\textsuperscript{2+}-uptake system \textit{znuABC} is regulated by the Fur family transcriptional regulator Zur (5). A homology survey displayed that the genome of the \textit{Xc} strain 8004 possesses a ZntA (\textit{Xc}3531 (GenBank accession number \textit{YP}244594)) and two ArsR/SmtB (\textit{Xc}1489 (GenBank accession number \textit{YP}242577) and \textit{Xc}2723 (GenBank accession number \textit{YP}243792)) homologues but not ZntR homologue. These facts, taken together, suggest that \textit{Xc} may have developed its specific zinc homeostasis machinery, the regulatory mechanism of which is distinct from that of \textit{E. coli}. A few members of the Fur family have been found to exhibit repressor and activator functions. For instance, the Fur of \textit{Neisseria meningitidis} (41) and \textit{Vibrio vulnificus} (42), the BosR of \textit{Borrelia burgdorferi} (43) and the PerR of \textit{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\textsuperscript{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 \textit{B. subtilis} positively regulates the expression of the genes \textit{rocD} and \textit{rocE} that are involved in amino acid transport (16), and the Zur of \textit{S. typhimurium} activates the expression of the virulence-related operon \textit{flhA} (9). Although it has been proposed that the \textit{B. subtilis} Zur may activate the expression of \textit{rocE} and \textit{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 responds 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 acts by directly binding to a 30-bp AT-rich sequence 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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