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.

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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 Zn\(^{2+}\) mobilization through regulating some Zn\(^{2+}\)-binding ribosomal proteins besides repressing Zn\(^{2+}\) uptake (12,13).

The molecular mechanism by which Zur represses the expression of Zn\(^{2+}\)-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 Zn\(^{2+}\) 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 the Zn\(^{2+}\) export gene, indicating that the Zur-binding sites of these Zur proteins may use a similar mechanism to repress the expression of the Zn\(^{2+}\)-uptake systems.

To the best of our knowledge, there is not report about a Zur to be involved in the regulation of Zn\(^{2+}\)-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 Zn\(^{2+}\)-uptake systems but also activates the expression of a gene encoding a Zn\(^{2+}\) 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 Zn\(^{2+}\)-export systems is distinct to that in the Zn\(^{2+}\)-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 | Reference or source |
|---------------------|-------------------------|---------------------|
| E. coli JM109       | *RecA1, endA1, gyrA96, thi, supE44, relA1 Δ (lac-proAB) F* | (32) Novagen, Germany |
|                     | [traD36, lacF, lacZ Δ M15] |                     |
| BL21(DE3)pLysS      | F*ompT1 bua8 (rg mu) gal dcm (DE3) pLysS | This work |
| BL1430              | BL21(DE3)pLysS harboring pET1430, Kan<sup>r</sup> |                     |
| Xcc                 | Wild-type, Rif<sup>r</sup> | (20) Novagen, Germany |
| 8004                | As 8004, but XC1430 (zur)∶pK18mob, Rif<sup>r</sup>, Kan<sup>r</sup>, Te<sup>c</sup> |                     |
| 1430nk              | As 8004, but XC2976∶pK18mob, Rif<sup>r</sup>, Kan<sup>r</sup> | This work |
| 2976nk              | 2976nk harboring pXC2976, Rif<sup>r</sup>, Kan<sup>r</sup>, Te<sup>c</sup> | This work |
| C2976              | 8004 containing plasmid pG0267, Rif<sup>r</sup>, Te<sup>c</sup> | This work |
| zurmt/pG0267        | 1430nk containing plasmid pG0267, Rif<sup>r</sup>, Kan<sup>r</sup>, Te<sup>c</sup> | This work |
| wt/pG02471-2        | 1430nk containing plasmid pG2471-2, Rif<sup>r</sup>, Kan<sup>r</sup>, Te<sup>c</sup> | This work |
| zurmt/pG2471-2      | 1430nk containing plasmid pG2471-2, Rif<sup>r</sup>, Kan<sup>r</sup>, Te<sup>c</sup> | This work |
| wt/pG2976           | 8004 containing plasmid pG2976, Rif<sup>r</sup>, Kan<sup>r</sup>, Te<sup>c</sup> | This work |
| zurmt/pG2976        | 1430nk containing plasmid pG2976, Rif<sup>r</sup>, Kan<sup>r</sup>, Te<sup>c</sup> | This work |
| wt/pG3788           | 8004 containing plasmid pG3788, Rif<sup>r</sup>, Kan<sup>r</sup>, Te<sup>c</sup> | This work |
| zurmt/pG3788        | 1430nk containing plasmid pG3788, Rif<sup>r</sup>, Kan<sup>r</sup>, Te<sup>c</sup> | This work |
| wt/pG3788MT         | 8004 containing plasmid pG2976MT, Rif<sup>r</sup>, Kan<sup>r</sup>, Te<sup>c</sup> | This work |
| Plasmids            | Expression vector, allow the production of fusion proteins containing amino terminal 6xHis-tagged sequences. Kan<sup>r</sup>, Sp<sup>c</sup>, Te<sup>c</sup> = rifampicin-, kanamycin-, spectinomycin-, and tetracycline-resistant, respectively. | Novagen, Germany |
| pET-30a-C(+)        | pET-30a-C(+) containing the coding region of the Xcc zur gene. Kan<sup>r</sup>, Sp<sup>c</sup>, Te<sup>c</sup> | This work |
| pET1430             | Broad host range cloning vector, Te<sup>c</sup> | (45) Novagen, Germany |
| pLAFR6              | Helper plasmid, Tra +, Mob +, ColEl, Spe′ | (46) Novagen, Germany |
| pK2073              | pLAFR6 containing a XCC0267 promoter-gusA fusion fragment, Te<sup>c</sup> | This work |
| pG2471-2            | pLAFR6 containing a XCC2472-XCC2471 promoter-gusA fusion fragment, Te<sup>c</sup> | This work |
| pG2976              | pLAFR6 containing a XCC2976 promoter-gusA fusion fragment, Te<sup>c</sup> | This work |
| pG3788              | pLAFR6 containing a XCC3788 promoter-gusA fusion fragment, Te<sup>c</sup> | This work |
| pG2976MT            | pLAFR6 containing a mutant XCC2976 promoter-gusA fusion fragment, Te<sup>c</sup> | This work |
| pX2976              | pLAFR6 containing a 1349-bp fragment including YCC2976 gene, Te<sup>c</sup> | This work |
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 (RT–PCR) and 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, 1.4M 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 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 ultrasoundicated for 30 min. The cell lysate was centrifuged at 4000 g 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 \(XCO267\), \(XCN2471\), \(XCN2976\) and \(XCN3788\), 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 \(gusA\) (\(\beta\)-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 plasmid 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 was 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 \(zur\)-mt/pG0267, \(zur\)-mt/pG2471-2, \(zur\)-mt/pG2976 and \(zur\)-mt/pG3788 (Table 1).

Site-directed mutagenesis of the \(XCN2976\) promoter

To mutate the \(XCN2976\) 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 \(XCN2976\)-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 \(XCN2976\) 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 \(XCN2976\) promoter fragment \(P_{2976\text{MT}}\), in which ACACCACACC was changed for the left half part of the 20 bp imperfect inverted repeat CGTGATGTGA of the wild-type \(XCN2976\) promoter, was obtained by PCR using pG2976MT DNA as template and the primer set \(P_{2976\text{F}}/P_{2976\text{R}}\) (Table S1).

GUS activity assay

The activity of \(\beta\)-glucuronidase (GUS) was determined after the growth of \(xcc\) strains in NYG, NYG supplemented with different levels of \(ZnSO_4\) or EDTA for 24 h by measurement of the OD415 using \(p\)-nitrophenyl \(\beta\)-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 \(XCO267\), \(XCN2471\), \(XCN2976\) and \(XCN3788\) or the intergenic region between \(XCN2471\) and \(XCN2472\) (IGR\(_{2471-2472}\), 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\(_{0267\text{F}}/P_{0267\text{R}}\), P\(_{2471-2\text{F}}/P_{2471-2\text{R}}\), P\(_{2976\text{F}}/P_{2976\text{R}}\), P\(_{3788\text{F}}/P_{3788\text{R}}\) and IGR\(_{2471-2\text{F}}/IGR_{2471-2\text{R}}\) (Table S1). After purified by using QIAquick PCR Purification Kit (Qiagen), these PCR products were labeled with \(^{[\gamma\text{-}32P]}\text{ATP}\) using a \(5\)-end labeled kit (Takara, Dalian, China). Then the His\(_{6}\)-Zur protein was added into a 1.5-ml EP tube containing 20 \(\mu\)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 \(\mu\)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 phosphorimagery screen (Typhoon 9410, Amersham Biosciences Corp., Piscataway, NJ USA).

To test the effects of \(Zn^{2+}\) and EDTA on protein–DNA binding, the His\(_{6}\)-Zur protein was added into 20 \(\mu\)l binding buffer and then \(ZnSO_4\) 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 \(XCO267\), \(XCN2471\), \(XCN2976\) and \(XCN3788\) 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 \(XCO267\), \(XCN2471\), \(XCN2976\) and \(XCN3788\), were used to amplify the corresponding promoter fragments for DNaseI footprinting analyses. The 5′-ends of the primers P\(_{0267\text{F}}\), P\(_{2471-2\text{F}}\), P\(_{2976\text{F}}\) and P\(_{3788\text{F}}\) were labeled with 5-carboxytetramethylrhodamine (Tamra) (Sangon, Shanghai, China). DNA fragments containing the promoter region of the ORFs \(XCO267\), \(XCN2471\), \(XCN2976\) and \(XCN3788\) 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 μl, and 5 μg of sheared salmon sperm DNA per μl] 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 0.15 M 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-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 (OD_600 ~ 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 dH_2O. 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 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 0267FtF/0267FtR, 2471-2tF/2471-2tR, 2976FtF/2976FtR and 3788FtF/3788FtR (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/2976MTR (Table S1) and then linked together to generate the P2976MT fragment by fusion PCR (26). Each promoter template fragment includes the promoter region as well as ~50-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 MgCl_2, 2 mM spermidine, 10 mM NaCl, 5 mM DTT, 5% glycerol, 50 mM KCl, 1U RNase inhibitor, 100 μM ZnSO_4] 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/μl)] 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 7 M 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 into 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 \(Xcc\) 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 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 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-\(\mu\)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 supplemented with a certain metal ion to a series of final concentrations, and the cell density for each treatment was adjusted using sterilized ddH2O to a final concentration of 300 \(\mu\)M and incubated for further 4 h. Cells from the treatment 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.

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 24 h. The levels of metal ions used in this study are \(ZnSO_4\): 0, 300, 400 and 500 \(\mu\)M; \(CoCl_2\): 0, 150, 200, 250, 300 and 350 \(\mu\)M; \(CdSO_4\): 0, 40, 50, 60, 70 and 80 \(\mu\)M and \(NiSO_4\): 0, 400, 500, 600, 700 and 800 \(\mu\)M.

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 \(\mu\)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 \(Xcc\) 2976, 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 \(Xcc\) 2976, the predicted products encoded by \(Xcc\) 2027, \(Xcc\) 2471 and \(Xcc\) 3788 displayed amino acid sequence similarity to the \(Zn^{2+}\) efflux-related gene and \(Zn^{2+}\) uptake-related proteins identified in other bacteria. \(Xcc\) 2471 and \(Xcc\) 2027 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 \(Zn^{2+}\)-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 \(Xcc\) 2976 is a putative \(Zn^{2+}\)-efflux-related gene and \(Xcc\) 2027 and \(Xcc\) 2471 as well as \(Xcc\) 3788 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 \(Xcc\) 2027, \(Xcc\) 2471, \(Xcc\) 3788 and \(Xcc\) 2976 in the genome of \(Xcc\) strain 8004 are shown in Table 2. \(Xcc\) 2027 and its adjacent ORF \(Xcc\) 2026 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
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 Xcc 8004's genome | Homology [Organism] (accession number) | Amino acid similarity (%) | Characterized function                                      | Reference |
|----------|--------------------------------|----------------------------------------|---------------------------|-------------------------------------------------------------|-----------|
| XCC0267  | 321454–322779                  | YcIC [B. subtilis] (CAB12130)          | 54                        | Component of the low-affinity Zn2+ uptake system YciABC     | (16)      |
| XCC2471  | 2989376–2988441                | YciA [B. subtilis] (CAB12128)          | 29                        | Component of the low-affinity Zn2+ uptake system YciABC     | (16)      |
| XCC2976  | 3563238–3562273                | CzcD [R. metallidurans] (YP_145596)   | 67                        | Acts as a Zn2+ efflux pump                                  | (33)      |
| XCC3788  | 4483267–4482527                | ZnuC [E. coli] (P0A9X1)                | 38                        | Component of the high-affinity Zn2+ uptake system ZnuABC    | (5)       |

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 XCC0267, XCC2471-2 and XCC3788 are repressed in Zn2+-rich condition and induced in Zn2+–limited condition. On the contrary, XCC2976 is induced in Zn2+-rich condition and repressed in Zn2+–limited condition. These further support the possibility that XCC0267, XCC2471 and XCC3788 may play roles in Zn2+ uptake-related function and XCC2976 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 XCC0267, XCC2471-2 as well as XCC3788 and the inducing effects on the expression of XCC2976 are mediated by Zur.

Zur activates the transcription of XCC2976 and represses XCC0267, XCC2471-2 and XCC3788 directly

The above studies have demonstrated that the Xcc Zur positively regulates the expression of XCC2976 and negatively regulates the expression of XCC0267, XCC2471-2 and XCC3788. 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)* |
|-----------------|-------------------|
| wt/pG0267       | 0.12 ± 0.02A      |
| zurmt/pG0267    | 1.78 ± 0.01C      |
| wt/pG2471-2     | 0.04 ± 0.001A     |
| zurmt/pG2471-2  | 0.21 ± 0.003C     |
| wt/pG3788       | 0.13 ± 0.01A      |
| zurmt/pG3788    | 4.04 ± 0.34C      |
| wt/pG2976       | 4.46 ± 0.10A      |
| zurmt/pG2976    | 0.49 ± 0.01C      |

*a*-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 (NYG + EGTA) or NYG supplemented with EGTA and Zn2+ to the final concentration of 0.5 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 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). 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) (AAGTGTGATATT) (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 TGGCCCAAAGCCTCTGATAATGTGGCCGCGCAC TACTCTAGTGTAGTGCAC) containing a 20-bp imperfect inverted repeat, GCACCTAATCT-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 Xc0266, 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-P0266, 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 ^{32}P-end-labeled DNA fragment in binding buffer.
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, CGTGTAGTGA, 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 His_{6}-tagged Zur protein. As shown in Figure 4B, the His_{6}-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,
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 μM ZnSO_4 for 4 h (Table 4). The complemented mutant strain contained similar amount of Zn^2+ as the wild-type (P<0.01). 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 μM 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).

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.biomedi.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 bacteria. To assess whether the czd homologous XC2976 is responsible for the resistance of Xcc to cobalt and cadmium, the sensitivity of the XC2976-mutant

Table 4. The intracellular zinc content of Xcc strains*

| Strains                               | Intracellular zinc content (µg/10^10 cells) |
|---------------------------------------|--------------------------------------------|
| 8004 (wild-type)                      | 1.4±0.11A                                  |
| 1430nk (zur mutant)                   | 6.2±0.21B                                  |
| 2976nk (XC2976 mutant)                | 6.4±0.16B                                  |
| C2976nk (complemented XC2976 mutant)  | 1.5±0.08A                                  |

*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.

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 −53 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 32P-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, pG2976) (b) or mutated Zur-binding site are shown. (30) and measured its intracellular Zn^2+ content as well as its growth rate in an excessive Zn^2+ medium (see Figure 5).
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 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 *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 homologue (XC3531 (GenBank accession number YP_244594)) and two ArsR/SmtB 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 meningitides* (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 *fliA* (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. 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 XCC2976 responses to Zn2+ through Zur mediation. The EMSA and DNaseI footprinting analyses demonstrate that the Xcc Zur binds to the promoter region of the Zn2+-export gene XCC2976. In vitro transcription assay demonstrates that Zur activates the transcription of XCC2976 directly. These results reveal that the Xcc Zur directly activates the expression of XCC2976. 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 XCC2976 promoter, which differs from the ~30-bp AT-rich sequence bound by Zur in the promoters of the Zn2+-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 XCC2976 promoter by Zur, indicating that the Xcc Zur positively regulates the transcription of XCC2976 by directly binding to a cis-acting element located in the XCC2976 promoter. How the Xcc Zur distinguishes these two different target sequences and what is the mechanism by which it activates the transcription of the Zn2+-export gene XCC2976 after binding to the promoter remain to be further investigated.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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