The Zinc Uptake Regulator Zur Is Essential for the Full Virulence of *Xanthomonas campestris* pv. *campestris*

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Zur is a regulator of the high-affinity zinc uptake system in many bacteria. In *Xanthomonas campestris* pv. *campestris* 8004, a putative protein encoded by the open reading frame designated as XC1430 shows 42% amino acid similarity with the Zur of *Escherichia coli*. An XC1430-disrupted mutant 1430nk was constructed by homologous suicide plasmid integration. 1430nk failed to grow in rich medium supplemented with Zn²⁺ at a concentration of 400 µM and in non-rich medium supplemented with Zn²⁺ at a concentration of 110 µM, whereas the wild-type strain grew well in the same conditions. In rich medium with 400 µM Zn²⁺, 1430nk accumulated significantly more Zn²⁺ than the wild-type strain. 1430nk showed a reduction in virulence on the host plant Chinese radish (*Raphanus sativus* L. var. *radiculus* Pers.) and produced less extracellular polysaccharide (EPS) than did the wild-type strain in the absence of added zinc. These results revealed that XC1430 is a functional member of the Zur regulator family that controls zinc homeostasis, EPS production, and virulence in *X. campestris* pv. *campestris*.

Zinc is an essential trace element for living organisms and functions as a structural or catalytic cofactor in numerous metalloproteins. However, zinc can be toxic if the intracellular concentration exceeds a critical level. As a consequence, the intracellular zinc level must be precisely regulated. Bacteria have developed both uptake and export systems that work together to maintain appropriate levels of zinc in the cell. In *Escherichia coli*, this task is accomplished by two zinc uptake systems (PitA and ZnuABC) and a zinc export system (ZntA) (Patzner and Hantke 1998). The ZnuABC system is negatively regulated in a zinc-dependent manner by the zinc-uptake regulatory protein Zur (Patzner and Hantke 1998). Zur proteins have been reported in *Bacillus subtilis* (Gaballa and Helmann 1998), *Staphylococcus aureus* (Lindsay and Foster 2001), *Listeria monocytogenes* (Dalet et al. 1999), and *Salmonella enterica* (Campoy et al. 2002). Zur also regulates additional functions to zinc uptake systems, including amino acid transport in *B. subtilis* (Gaballa et al. 2002) and the expression of the virulence-related gene *fliAZ* in *Salmonella typhimurium* (Campoy et al. 2002).

Although described for bacterial pathogens of animals and humans, the involvement of Zur in the virulence of plant pathogens has not been examined (Campoy et al. 2002; Patzner and Hantke 1998; Wang et al. 1996). In this paper, we report the identification of a gene encoding a Zur-like protein in *X. campestris* pv. *campestris*, the causal agent of black rot disease of cruciferous plants (Williams 1980) and its role in zinc homeostasis and virulence.

RESULTS

Proteins deduced from *Xanthomonas* genomic sequences with homology to zinc uptake regulatory proteins.

The complete genome sequences of *X. campestris* pv. *campestris* strains ATCC33913 (da Silva et al. 2002) and 8004 (W. Qian, S.-X. Ren, D.-J. Tang, Y.-Q. He, B. Chen, G.-P. Zhao, C. He, and J.-L. Tang, *unpublished data*) contain a gene encoding a protein of 172 amino acid residues that shares 42 and 40% amino acid similarity with the Zur proteins of *E. coli* (GenBank accession number P32692) and *S. typhimurium* (accession number AAL56650), respectively (Fig. 1). The gene products from the two *X. campestris* pv. *campestris* strains, open reading frames (ORF) XCC2685 (ATCC33913) and XC1430 (8004) are identical. XC1430 and the upstream gene XC1429 are oriented in the same direction separated by 13 bp between the stop codon of XC1429 and the start codon of XC1430 (Fig. 2). For complementation, pXC1430 (Table 1) was constructed by cloning an 833-bp fragment from 114 bp upstream of the start codon to 200 bp downstream of the stop codon of XC1430 into the promoterless pLAFR6 (Fig. 2). Although XC1430 may be the last ORF of a polycistronic operon, pXC1430 was functional for the phenotypic assays, indicating that the 833-bp fragment contains a functional promoter. We further found that a shorter 785-bp fragment that includes the region from 66 bp upstream of the start codon to 200 bp downstream of the stop codon of XC1430 was also functional (data not shown), further indicating a functional promoter of XC1430 is located in the region of –66 to –24 (Fig. 2).

XC1430 is involved in zinc homeostasis.

To determine the function of the gene in *X. campestris* pv. *campestris*, XC1430 was disrupted by homologous suicide plasmid integration, resulting in 1430nk, and the effect of the mutation on zinc homeostasis was examined. No significant difference in growth was observed between 1430nk and the

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Nucleotide sequence data is available in the GenBank database under accession number AY626795.

wild-type strain 8004 in rich medium (NYGB) without supplementation of zinc or defined minimal medium XVM2 without supplementation of zinc (Fig. 3). Differences in growth between 8004 and 1430nk were observed in the presence of increasing levels of zinc. 8004 could grow well in NYGB supplemented with concentrations up to 400 μM ZnSO_4 and, in XVM2, up to 110 μM ZnSO_4 , whereas the mutant 1430nk failed to grow under the same conditions (Fig. 3). The ability to tolerate high zinc levels was restored by pXC1430 (Fig. 3). These results indicate that XC1430 is required for zinc tolerance of *X. campestris* pv. *campestris*. The zinc content of 1430nk was significantly higher than that of 8004 when grown in NYGB supplemented with 300 μM of ZnSO_4 (Table 2), indicating that XC1430 is required for zinc-uptake regulation. The intracellular zinc level of 1430nk was also restored to the wild-type level by pXC1430 (Table 2). No significant difference in the sensitivity of 1430nk and 8004 was observed in NYGB or XVM2 media supplemented with different concentrations of FeSO_4 and MgSO_4 , demonstrating that the mutation in XC1430 did not have a general effect on the sensitivity to divalent metal ions.

XC1430 is essential

for full virulence of *X. campestris* pv. *campestris*.

The virulence of 1430nk was tested on Chinese radish (*Raphanus sativus* L. var. *radiculus* Pers.) by the leaf-clipping method. The mutant showed a reduction in virulence, as compared with the wild-type strain, that could be fully restored by pXC1430 (Fig. 4). To test the role of XC1430 in growth of *X. campestris* pv. *campestris* in the host, the populations of 1430nk and 8004 in radish leaves were determined. The bacterial number of 1430nk recovered from the infected leaves was approximately 10-fold lower than that of the wild-type strain 5 days postinoculation and onward (Fig. 5). The growth capacity of the mutant in planta could be completely restored by pXC1430 (Fig. 5).

XC1430 is required

for the EPS production of *X. campestris* pv. *campestris*.

Extracellular polysaccharide (EPS) and extracellular enzymes are important virulence factors for *X. campestris* pv. *campestris* (Chan and Goodwin 1999). To determine whether the mutation in XC1430 has any effect on the production of

Fig. 1. Alignment of amino acid sequences of Zur homologs from *Xanthomonas campestris* pv. *campestris* and *Escherichia coli*. The *E. coli* Zur sequence (GenBank accession number P32692) was from the sequence databases on the National Center for Biotechnology Information website. Black boxes denote identical amino acid residues, whereas gray-shaded boxes are similar between the two sequences.

Fig. 2. Schematic presentation of the DNA fragment used for XC1430 mutant complementation. The far left of the fragment reached into the coding region of XC1429 by 100 bp, and the far right was beyond the stop codon of XC1430 by 200 bp. The putative promoter region and the putative ribosomal binding site (RBS) are underlined.

these factors, the EPS production and the activities of the extracellular protease, amylase, and endoglucanase were measured. 1430nk had lower levels of EPS production than 8004 (Table 2), while no differences were detected in the production of extracellular enzymes (data not shown). The EPS production of the mutant could be restored to the wild-type level by pXC1430 (Table 2). To investigate if the Zn^{2+} concentration in the medium influences EPS production, the EPS yield was measured when the bacterial strains were grown in media with different levels of Zn^{2+} . No difference was observed in the EPS yield of either the wild-type strain or 1430nk (Table 3).

The results indicated that XC1430 may regulate EPS production of *X. campestris* pv. *campestris*. To establish the regulatory relationship between *zur* and EPS production, the regulation of the EPS-related genes *clp* (de Crecy-Lagard et al. 1990), *gumB* (Katzen et al. 1996), *opsX* (Kingsley et al. 1993), and *rpfC* (Tang et al. 1991) was examined by measuring the β -glucuronidase (GUS) activities of *clp-gus*, *gumB-gus*, *opsX-gus*, and *rpfC-gus* fusion reporter genes in 1430nk and 8004. The reporters were generated by Tn5gusA5 transposon-mediated insertion mutagenesis in which the promoterless *uidA* was fused to the target ORF. These mutants include 167D11 (*gumB*⁻), 115G10 (*clp*⁻), 101B06 (*opsX*⁻) and 124B03 (*rpfC*⁻) (Table 1). GUS activities of these mutants were not affected by XC1430 mutation, indicating that the *zur* may not regulate these EPS production-related genes (Table 4).

DISCUSSION

Sequence analysis of ORF XC1430 of *X. campestris* pv. *campestris* 8004 indicated that the protein product is a possible homolog of the *zur* gene. A mutation in this gene resulted in an increase in sensitivity specifically to zinc, and the mutant strain accumulated more zinc than did the wild-type strain when grown in the high-zinc medium. However, the mutant did not show similar sensitivity to the change of magnesium and iron concentration. These findings established a role for XC1430 in zinc homeostasis and strongly support the contention that XC1430 encodes a Zur protein.

The similarity of Zur from *X. campestris* pv. *campestris* and *E. coli* is 40%. Five *Xanthomonas* species have been sequenced,

and a BLAST search with XC1430 against these genomes revealed a Zur-like protein in *X. campestris* pv. *campestris* ATCC33913 (ORF XCC2685; 100% identity), *X. axonopodis* pv. *citris* 306 (XAC2846; 94%), *Xylella fastidiosa* 9a5c (XF0821, 83%), and *Xylella fastidiosa* Temecula1 (PD1849, 80%). Whether these latter proteins are involved in zinc homeostasis is unknown. However, the results with XC1430 and the close similarity of the ORF indicate that these gene products are also likely to function as Zur proteins. Zur negatively regulates the expression of *znuABC*, the high-affinity zinc uptake system in *E. coli* (Patzer and Hantke 1998). Genes with clear similarity to *znuABC* were not detected in *X. campestris* pv. *campestris*, suggesting that these genes may be highly divergent. However, it is reasonable to assume that *znuABC* isofunctional genes exist in *X. campestris* pv. *campestris*, based on the excessive accumulation of zinc in 1430nk.

Interestingly, the reduction in virulence of the *zur* mutant of *X. campestris* pv. *campestris* does not appear to be due to zinc toxicity. The *zur* mutant could grow normally in either complete media supplemented with up to 400 μ M Zn^{2+} and defined media supplemented with 110 μ M Zn^{2+} . Zn^{2+} concentration in the intercellular fluid extract of the host leaf is at a level similar to that in NYGB medium (11.2 μ M in the intercellular fluid extract and 9.7 μ M in NYGB medium) (D.-J. Tang, X.-J. Li, Y.-Q. He, J.-X. Feng, B. Chen, and J.-L. Tang, *unpublished results*). We also compared the growth rates of the *zur* mutant with those of the wild-type strain in medium supplemented with different levels of EGTA (ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid), the Zn^{2+} chelator, and found no differences, indicating that reduced virulence of the *zur* mutant is not due to a Zn^{2+} deficiency inside the host (data not shown).

We speculate that the attenuation in virulence and reduction in growth in planta of the *zur* mutant is the result of the altered expression of virulence genes, yet to be identified, that are regulated by Zur. Evidence from other bacteria indicates that Zur is a multifunctional regulatory protein regulating the expression of *rocE* and *rocD*, which are involved in amino acid transport in *B. subtilis* (Gaballa et al. 2002), and the expression of the virulence-related gene *fliZ* in *S. enterica* serovar *typhimurium* (Campoy et al. 2002), in addition to genes involved in zinc uptake systems. Disruption of *zur* in *S. enterica* serovar

Table 1. Bacterial strains and plasmids used in this work^z

Strains or plasmids	Relevant characteristics	Reference or source
<i>Escherichia coli</i>		
JM109	<i>RecA1</i> , <i>endA1</i> , <i>gyrA96</i> , <i>thi</i> , <i>supE44</i> , <i>relA1</i> $\Delta(lac-proAB)/F'$ [<i>traD36</i> , <i>lacI</i> ^h , <i>lacZ</i> Δ M15]	Yanisch-Perron et al. 1985
<i>Xanthomonas campestris</i> pv. <i>campestris</i>		
8004	Wild type, Rif ^r	Daniels et al. 1984
1430nk	As 8004, but XC1430::pK18mob, Rif ^r , Kan ^r	This study
167D11	As 8004, but <i>gumB</i> ::Tn5gusA5, Rif ^r , Kan ^r , Spc ^r , Gm ^r	Laboratory collection
115G10	As 8004, but <i>clp</i> ::Tn5gusA5, Rif ^r , Kan ^r , Spc ^r , Gm ^r	Laboratory collection
101B06	As 8004, but <i>opsX</i> ::Tn5gusA5, Rif ^r , Kan ^r , Spc ^r , Gm ^r	Laboratory collection
124B03	As 8004, but <i>rpfC</i> ::Tn5gusA5, Rif ^r , Kan ^r , Spc ^r , Gm ^r	Laboratory collection
167D11Z	As 167D11, but XC1430::pT18mob, Rif ^r , Kan ^r , Spc ^r , Gm ^r , Tc ^r	This study
115G10Z	As 115G10, but XC1430::pT18mob, Rif ^r , Kan ^r , Spc ^r , Gm ^r , Tc ^r	This study
101B06Z	As 101B06, but XC1430::pT18mob, Rif ^r , Kan ^r , Spc ^r , Gm ^r , Tc ^r	This study
124B03Z	As 124B03, but XC1430::pT18mob, Rif ^r , Kan ^r , Spc ^r , Gm ^r , Tc ^r	This study
C1430nk	1430nk harboring pXC1430, Rif ^r , Kan ^r , Tc ^r	This study
Plasmids		
pLAFR6	Broad-host range cloning vector, Tc ^r	Huynh et al. 1989
pRK2073	Helper plasmid, Tra ⁺ , Mob ⁺ , ColE1, Spc ^r	Leong et al. 1982
pK18mob	Suicide plasmid in <i>X. campestris</i> pv. <i>campestris</i> , Kan ^r	Schafer et al. 1994
pT18mob	A tetracycline-resistant derivative of pK18mob, Tc ^r	Laboratory collection
pK1430	pK18mob containing a 400-bp internal fragment of XC1430, Kan ^r	This study
pT1430	pT18mob containing a 400-bp internal fragment of XC1430, Kan ^r	This study
pXC1430	pLAFR6 containing a 833-bp fragment including XC1430 gene, Tc ^r	This study

^z Rif^r, Kan^r Spc^r, Gm^r, Tc^r = rifampicin-, kanamycin-, spectinomycin-, gentamicin-, and tetracycline-resistant, respectively.

typhimurium resulted in an approximately 10-fold increase in the LD₅₀ (lethal dose, 50%) to mice. The decrease in virulence could be related to the decreased expression of *fliAZ*, which positively regulates the invasion genes (Iyoda et al. 2001). We could not find a *fliZ* homolog in the genome of *X. campestris* pv. *campestris*. Zur is also involved in the virulence of the animal pathogen *Pseudomonas aeruginosa* (Patzner and Hantke 1998; Wang et al. 1996).

The mutation in *zur* of *X. campestris* pv. *campestris* did not affect the production of extracellular enzymes (protease, amylase, and endoglucanase) or the ability to elicit a hypersensitive response in a nonhost plant species (data not shown), suggesting that Zur is not involved in regulation of the expression of type II or type III secretion systems. However, a Zur mutation did alter the production of EPS in *X. campestris* pv. *campestris*. EPS has been identified as an important virulence factor of *X. campestris* pv. *campestris* (Chan and Goodwin 1999), and it is logical to assume that the reduction in EPS may contribute, at least in part, to the attenuation in virulence of the *zur* mutant. EPS production in *X. campestris* pv. *campestris* is under a complex control, including regulation by *clp*, *rpf*, and *opaX* (de Crecy-Lagard et al. 1990; Kingsley et al. 1993; Tang et al. 1991). However, none of these genes are regulated by Zur under the conditions used in this study. Thus,

Zur must exert its regulatory effect on EPS production through a route yet to be identified. It is also possible that Zur regulates the expression of other virulence genes, a topic that merits further investigation.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions.

Bacteria and plasmids used in this work are listed in Table 1. *E. coli* strains were grown in Luria-Bertani medium (Miller 1972) at 37°C. *X. campestris* pv. *campestris* strains were grown in NYGB medium (5 g of peptone, 3 g of yeast extract, and 20 g of glycerol per liter) (Daniels et al. 1984) or

Table 2. Zinc content and extracellular polysaccharide (EPS) production by *Xanthomonas campestris* pv. *campestris* strains²

Strains	Zinc contents (µg/10 ¹⁰ cells)	EPS yields (g/liter)
8004 (wild type)	1.67 ± 0.07 a	9.32 ± 0.55 a
1430nk (<i>zur</i>)	4.21 ± 0.36 b	2.77 ± 0.86 b
C1430nk (complemented mutant)	1.83 ± 0.12 a	9.47 ± 0.60 a

² Data are the mean ± standard deviation of triplicate measurements; different letters in each data column indicate significant differences at *P* = 0.05.

Fig. 3. XC1430 is required for zinc tolerance for *Xanthomonas campestris* pv. *campestris*. Overnight culture (200 µl, cell density adjusted to an optical density at 600 nm of 1.0) of each *X. campestris* pv. *campestris* strain was inoculated into 200 ml of rich medium (NYGB) or XVM2 medium supplemented with ZnSO₄ to a final concentration of 0, 200, 300, 400, 500, or 600 µM in NYGB and 0, 50, 70, 90, 110, or 130 µM in XVM2. Cells grown **A**, in NYGB were incubated at 28°C with shaking at 200 rpm for 24 h, and cells grown **B**, in XVM2 were incubated at 28°C with shaking at 200 rpm for 72 h. The cell density was measured spectrometrically at 600 nm. Values are the mean ± standard deviation from three repeats.

XVM2 medium (20 mM NaCl, 10 mM $[\text{NH}_4]_2\text{SO}_4$, 5 mM MgSO_4 , 1 mM CaCl_2 , 0.16 mM KH_2PO_4 , 0.32 mM K_2HPO_4 , 0.01 mM FeSO_4 , 10 mM fructose, 10 mM sucrose, 0.03% casamino acids) (Wengelnik et al. 1996) at 28°C. Antibiotics were added to the media at the following final concentrations: rifampicin, 50 µg/ml; kanamycin, 25 µg/ml; ampicillin, 100 µg/ml; and tetracycline, 15 µg/ml for *E. coli* and 5 µg/ml for *X. campestris* pv. *campestris*. The genome sequence of *X. campestris* pv. *campestris* 8004 has been determined by a collaborative effort involving our laboratory (W. Qian, S.-X. Ren, D.-J. Tang, Y.-Q. He, B. Chen, G.-P. Zhao, C. He, and J.-L. Tang, unpublished data).

Fig. 4. XC1430 is essential for full virulence of *Xanthomonas campestris* pv. *campestris*. **A**, Symptoms on Chinese radish leaves caused by *X. campestris* pv. *campestris* strains. Photographs were taken 10 days after inoculation. **B**, The average lesion lengths caused by *X. campestris* pv. *campestris* strains. Value is the mean $\pm$ standard deviation from three repeats, each with 50 leaves.

Table 3. Effect of Zn^{2+} concentration on extracellular polysaccharide (EPS) production of *Xanthomonas campestris* pv. *campestris* strains^a

Strains	EPS yield ^a			
	0 µM	100 µM	150 µM	200 µM
8004 (wild type)	9.1 $\pm$ 0.38a	8.5 $\pm$ 0.29a	8.6 $\pm$ 0.28a	8.3 $\pm$ 0.38a
1430nk (<i>zur</i> mutant)	2.5 $\pm$ 0.16b	2.3 $\pm$ 0.20b	2.6 $\pm$ 0.11b	2.2 $\pm$ 0.26b

^a EPS = extracellular polysaccharide. Data are the mean $\pm$ standard deviation of triplicate measurements; different letters in each data column indicate significant differences at $P = 0.05$.

^a In grams per liter in medium supplemented with different levels of ZnSO_4 .

DNA manipulations.

DNA manipulations followed the procedures described by Sambrook and associates (1989). The conjugation between *X. campestris* pv. *campestris* and *E. coli* strains was performed as described by Turner and associates (1985). Restriction enzymes and DNA ligase were used in accordance with the manufacturer's instructions (Promega, Shanghai).

Construction of an XC1430 mutant.

XC1430 was inactivated by homologous suicide plasmid integration as described by Windgassen and associates (2000), using pK18mob as a vector (Schafer et al. 1994). A 400-bp internal fragment of the XC1430 gene was amplified using the total DNA of *X. campestris* pv. *campestris* 8004 as template and the primer set 1430MF (5'-CAGGGATCCATGCCAACA GCTTCGTACGTGCGGT-3') and 1430MR (5'-GGGAAGCT TGTCTGTGCCTGCGGTGGAAGCCCA-3'). After confirmation by sequencing, the amplified DNA fragment was cloned into pK18mob to create the recombinant plasmid pK1430. The

Fig. 5. Growth of *Xanthomonas campestris* pv. *campestris* strains in Chinese radish leaves. Five inoculated leaves for each strain were taken daily and were homogenized in sterile water. Diluted homogenates were plated on rich medium supplemented with rifampicin (for the wild-type strain) and rifampicin plus kanamycin (for mutants). Bacterial CFU were counted after incubation at 28°C for 3 days and were expressed as CFU per leaf. Data are the mean $\pm$ standard deviation from three repeats. ◇ represents 8004, ■ 1430nk, and ▲ C1430nk.

Table 4. Effect of mutation in XC1430 on the expression of genes involved in extracellular polysaccharide production of *Xanthomonas campestris* pv. *campestris* strains^a

Strains	GUS activities (U)
167D11 (<i>gumB::gusA</i>)	2.757 $\pm$ 0.208
167D11Z (<i>gumB::gusA</i> -XC1430::pT18mob)	2.200 $\pm$ 0.182
115G10 (<i>clp::gusA</i>)	1.039 $\pm$ 0.109
115G10Z (<i>clp::gusA</i> -XC1430::pT18mob)	1.039 $\pm$ 0.110
101B06 (<i>opsX::gusA</i>)	0.541 $\pm$ 0.089
101B06Z (<i>opsX::gusA</i> -XC1430::pT18mob)	0.377 $\pm$ 0.031
124B03 (<i>rpfC::gusA</i>)	0.405 $\pm$ 0.168
124B03Z (<i>rpfC::gusA</i> -XC1430::pT18mob)	0.363 $\pm$ 0.045

^a β -Glucuronidase (GUS) activities were determined after growth of *X. campestris* pv. *campestris* strains in rich medium plus 2% glucose for 24 h. Data are the mean $\pm$ standard deviation of triplicate measurements.

pK1430 was introduced from *E. coli* JM109 (Yanisch-Perron et al. 1985) into *X. campestris* pv. *campestris* 8004 by triparental conjugation using pRK2073 (Leong et al. 1982) as the helper plasmid. Transconjugants were screened on NYGA (NYGB supplemented with 1.5% agar) supplemented with rifampicin and kanamycin, and the obtained transconjugants with a mutation in the XC1430 gene were confirmed by polymerase chain reaction (PCR). Confirmation PCR was performed by using the total DNA of the obtained transconjugants as template and the primers P18conF (5'-GCCGATTCATTAATGCAGCTGGCAC-3'), which is located in pK18mob, and 1430conR (5'-CGATTGTTTAGCGTCTTGCGCGGGC-3'), which is located in downstream of the 400-bp internal fragment, with total DNA of *X. campestris* pv. *campestris* 8004 as the negative control. The expected PCR products were further confirmed by sequencing analysis. One of the confirmed mutants, named 1430nk, was used for further study.

Double mutant construction.

Strains 167D11, 115G10, 101B06, and 124B03 are the transposon Tn5gusA5 insertional mutants of *X. campestris* pv. *campestris* 8004. In the mutants, the promoterless *gus* gene is fused in the same transcriptional direction as the target gene. To inactivate the XC1430 in these mutant strains, a 400-bp internal fragment of the XC1430 gene, amplified using the total DNA of *X. campestris* pv. *campestris* 8004 as template and primer set 1430MF and 1430MR as primers, was cloned into pT18mob (Table 1) to create the recombinant plasmid pT1430. The plasmid pT1430 was introduced from *E. coli* JM109 into *X. campestris* pv. *campestris* strains 167D11, 115G10, 101B06, and 124B03 by triparental conjugation using pRK2073 as the helper plasmid. Transconjugants were screened on NYGA supplemented with rifampicin, kanamycin, and tetracycline. The obtained transconjugants with a mutation in the XC1430 gene were confirmed by PCR. Confirmation PCR was performed using the total DNA of the obtained transconjugants as template and primer set P18conF and 1430conR as primers, and total DNA of corresponding parent strain 167D11, 115G10, 101B06, or 124B03 were used as negative control. The expected PCR products were further confirmed by sequencing analysis. The resulting double mutants were named, respectively, 167D11Z, 115G10Z, 101B06Z, and 124B03Z (Table 1).

Complementation of 1430nk.

An 833-bp DNA fragment flanking the XC1430 coding region and extending 114 bp at the 5' and 200 bp at the 3' (Fig. 2) was amplified by PCR, using total DNA of *X. campestris* pv. *campestris* 8004 as template and primer set 1430F (5'-CCCGGATCCCCAGGTCAGCCCGGACATTTCGC-3') and 1430R (5'-ACTAAGCTTCCACTAGCAGCGGCTCGATTCCGC-3') as primers. After confirmation by sequencing analysis, the amplified DNA fragment was cloned into pLAFR6 (Huynh et al. 1989) to generate recombinant plasmid pXC1430 (Table 1). The plasmid was transferred into the mutant 1430nk by triparental conjugation. The transconjugants carrying the recombinant plasmid were screened on NYGA with rifampicin, kanamycin, and tetracycline and were named C1430nk (Table 1).

Metal ion sensitivity tests.

To test for Zn²⁺ sensitivity, 200 µl of overnight culture of *X. campestris* pv. *campestris* strains with an optical density at 600 nm of 1.0 (OD₆₀₀ = 1.0) was inoculated into 200 ml of NYGB, supplemented with ZnSO₄ to final concentrations of 0, 200, 300, 400, 500, or 600 µM or with XVM2 medium to final concentrations of 0, 50, 70, 90, 110, or 130 µM, and

was incubated at 28°C with shaking at 200 rpm. After incubation for 24 h in NYGB or 48 h in XVM2, the cell density was measured spectrometrically at 600 nm. For Mg²⁺ and Fe²⁺ sensitivity tests, 2 µl of overnight culture of bacterial strains with OD₆₀₀ = 1.0 was spotted onto NYGA or XVM2 plates supplemented with FeSO₄ or MgSO₄ to a series of final concentrations, respectively, and was incubated at 28°C for 48 h.

Measurement of zinc content.

For measurement of zinc content in *X. campestris* pv. *campestris* cells, 200 µl of overnight cultures of the bacterial strains were inoculated into 200 ml NYGB medium. After incubation at 28°C with shaking at 200 rpm for 12 h, the cultures were supplemented with 300 µM of ZnSO₄ and were further incubated for 4 h. Cells from the cultures were harvested and were washed twice with washing solution containing 0.1 M LiCl, 0.2 mM EDTA, and 0.1 mM EGTA to remove externally bound Zn²⁺. The cell density was adjusted using sterilized double distilled water to an OD₆₀₀ = 1.0, and the cells were kept on ice. The zinc content in the cells was determined with a Hitachi Model Z-7000 atomic absorption spectrophotometer (Hitachi Ltd., Marunouchi, Tokyo, Japan).

The zinc content in NYGB and XVM2 medium, and the intercellular fluid extracts of leaves of Chinese radish also was determined using the same instrument.

Test of extracellular enzymes and polysaccharide production.

Protease activity was detected on milk agar plates as described by Tang and associates (1987). The endoglucanase and amylase activity assay was performed as described by Slater and associates (2000). For EPS measurements, cultures were grown in NYGB medium containing 2% glucose at 28°C with shaking at 200 rpm for 5 days. EPS was precipitated from the culture supernatant with ethanol and was dried and weighed as described by Tang and associates (1991).

Virulence assay and determination of bacterial load in planta.

Virulence was tested on potted Chinese radish (*Raphanus sativus* L. var. *radiculus* Pers.) growing in a greenhouse with 12-h day and night cycle illuminations by fluorescent lamp at temperatures of 25 to 28°C. Seedlings with four fully expanded leaves were used for inoculation. Bacterial cells were grown in NYGB medium at 28°C with shaking at 200 rpm for 15 h. Cell concentration was adjusted to OD₆₀₀ = 1.0. Two to three leaves per plant were inoculated by leaf clipping. For leaf clipping, fully expanded leaves were cut with scissors dipped in the bacterial suspensions. A total of 60 to 80 leaves were inoculated in each independent experiment. Each treatment was repeated three times. After maintenance at 100% humidity for 24 h, the inoculated plants were maintained in the growth conditions described above. Lesion length was measured 10 days postinoculation.

The growth of bacteria in radish leaf tissue was measured by homogenizing a group of leaves (five leaves for each sampling) in 9 ml of sterile water. Diluted homogenates were plated on NYGA supplemented with rifampicin (for the wild-type strain) or rifampicin plus kanamycin (for mutants). Bacterial colony-forming units were counted after incubation at 28°C for 3 days.

GUS activity assay.

GUS activities were determined after growth of *X. campestris* pv. *campestris* strains in NYGB supplemented with 2% glucose for 24 h by measurement of the OD₄₁₅, using p-nitro-

phenyl β -D-glucuronide as substrate, as described by Henderson and associates (1985).

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