The Zur of *Xanthomonas campestris* Is Involved in Hypersensitive Response and Positively Regulates the Expression of the *hrp* Cluster Via *hrpX* But Not *hrpG*

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Submitted 22 July 2008. Accepted 13 November 2008.

In bacteria, Zur is a key regulator for zinc homeostasis. Our previous work has shown that, in the phytopathogen *Xanthomonas campestris* pv. *campestris*, in addition to regulating zinc homeostasis, Zur is essential for full virulence. Here, we demonstrate that the *X. campestris* pv. *campestris* Zur is involved in hypersensitive response (HR) and positively regulates the transcription of *hrpA* to *hrpF* operons and *hrpX* but not *hrpG*. Constitutively expressing *hrpX* but not *hrpG* in the *zur* mutant could bypass the requirement of Zur for the expression of *hrpA* to *hrpF* operons and the induction of wild-type HR, indicating that Zur controls the expression of *hrp* cluster via *hrpX*. Promoter-*gusA* reporter and semiquantitative reverse-transcription polymerase chain reaction analyses revealed that HrpG controls the expression of *hrpX* and HrpX regulates the expression of all the six *hrp* operons (*hrpA* to *hrpF*) in *X. campestris* pv. *campestris*.

Hypersensitive response (HR) is a disease-resistant phenomenon with a type of rapid, localized, and programmed cell death, which is induced by a pathogen at the infection sites of resistant hosts and nonhost plants (Alfano and Collmer 2004; Jones and Dangl 2006). A number of genes associated with HR and pathogenicity (*hrp*) in phytopathogenic bacteria have been characterized and most of them cluster on one chromosome (Alfano and Collmer 1997; Büttner and Bonas 2006; Lindgren 1997). Most of the *hrp* cluster genes encode the type III secretion system (T3SS) that is highly conserved among gram-negative pathogenic bacteria and translocates effector proteins into host cells (Cornelis and Van Gijsegem 2000; He 1998; Hueck 1998). In *Xanthomonas* spp., the *hrp* cluster consists of six operons (*hrpA* to *hrpF*) which harbor more than 20 different genes (Arlat et al. 1991; Bonas et al. 1991; Büttner et al. 2003; Gürlbeck et al. 2006). The expression of these *hrp*

genes is controlled positively by two key regulators, *hrpG* and *hrpX*, whose loci are far away from the *hrp* cluster on the chromosome (Büttner and Bonas 2006; Weber et al. 2007). HrpG is predicted to be an OmpR family's response regulator of two-component signal transduction systems, and HrpX is an AraC-type transcriptional activator. In *Xanthomonas campestris* pv. *vesicatoria*, the causal agent of bacterial spot disease in pepper and tomato, HrpG and HrpX form a regulatory cascade, in which HrpG regulates the expression of the *hrpA* operon and *hrpX*, and HrpX then activates the expression of other *hrp* operons (*hrpB* to *hrpF*) (Wengelnik and Bonas 1996; Wengelnik et al. 1996b). Although the *hrp* clusters among *Xanthomonas* spp. are highly conserved (Büttner et al. 2003; Gürlbeck et al. 2006), the regulatory cascade controlling expression of the *hrp* operons in different species or pathovars other than *X. campestris* pv. *vesicatoria* has not been experimentally verified.

In bacteria, the zinc uptake regulator Zur usually functions as a repressor of Zn^{2+} uptake systems to maintain zinc homeostasis (Hantke 2001, 2005). Recently, we have demonstrated that, in addition to playing a role in zinc homeostasis, the Zur of *X. campestris* pv. *campestris* is essential for full virulence (Tang et al. 2005) and also acts as an activator of a Zn^{2+} efflux pump, apart from acting as a repressor of Zn^{2+} uptake systems (Huang et al. 2008). *X. campestris* pv. *campestris* is the causal agent of black rot disease of cruciferous crops worldwide (Hayward 1993). The pathogen attacks not only economically important cruciferous vegetables and the major oil crop rape but also the model plant *Arabidopsis thaliana*. In the last more than two decades, it has been used as a model bacterium for studying plant–pathogen interactions, and a large number of *hrp* genes and genes implicated in virulence have been characterized (Arlat et al. 1991; Daniels et al. 1984a; Dow et al. 2003; He et al. 2007b; Qian et al. 2005; Tang et al. 1991). In this article, we described that the Zur of *X. campestris* pv. *campestris* is a novel key regulator for the expression of *hrp* genes, which positively regulates the expression of the *hrp* cluster through *hrpX*. Furthermore, we demonstrated that, in *X. campestris* pv. *campestris*, HrpX activates not only the expression of *hrpB* to *hrpF* operons but also the expression of *hrpA*.

RESULTS

The Zur of *X. campestris* pv. *campestris* is involved in HR.

Our previous work showed that, 24 h after inoculation, the *zur* mutant 1430nk (Table 1) induced a wild-type HR in a non-host local pepper cultivar inoculated by infiltrating bacterial

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*The e-Xtra logo stands for “electronic extra” and indicates that a supplemental table is available online.

suspension with a cell concentration of 1×10^8 CFU/ml (Tang et al. 2005). To evaluate the role of the *X. campestris* pv. *campestris* Zur in HR precisely, in this study, we investigated the HR of the *zur*-mutant strain 1430nk on the pepper cv. ECW-10R (*Capsicum annuum* cv. ECW-10R), one of the pepper cultivars typically used to test the HR of *X. campestris* pv. *campestris* (Castañeda et al. 2005; Newman et al. 2001). The results showed that the *zur* mutant 1430nk and the wild-type strain 8004 elicited almost identical HR symptoms 16 h after inoculation (Fig. 1). However, 8 h after inoculation, the *zur* mutant 1430nk was unable to trigger a visual HR on the inoculated leaves, whereas the wild type induced normal HR symptoms (Fig. 1). The complemented strain C1430nk (Table 1) (i.e., the mutant 1430nk harbors an entire *X. campestris* pv. *campestris zur* cloned in the broad-host-range vector pLAFR6) (Huynh et al. 1989) stimulated a wild-type HR 8 h after inoculation (Fig. 1), indicating that the HR-induction capacity of the *zur* mutant could be completely restored by *zur* in trans. These results demonstrate that *zur* is involved in HR induction of *X. campestris* pv. *campestris*.

Zur regulates positively the expression of *hrp* operons (*hrpA* to *hrpF*) and *hrpX* but not *hrpG*.

To determine whether *zur* has any regulatory relations with *hrp* genes, the plasmid-driven promoter β -glucuronidase (*gusA*) transcriptional fusion reporter plasmids pGUS*hrpA*, pGUS*hrpB*, pGUS*hrpC*, pGUS*hrpD*, pGUS*hrpE*, pGUS*hrpF*, pGUS*hrpG*, and pGUS*hrpX* (Table 1), in which a DNA fragment containing the promoter region of each of the six *hrp* operons (*hrpA* to *hrpF*) and the two key *hrp* regulators (*hrpG* and *hrpX*) of *X. campestris* pv. *campestris* fused to the promoterless *gusA* gene with its ribosome binding site (RBS) was cloned into the vector pLAFR6, were introduced from the *Escherichia coli* JM109 (Yanisch-Perron et al. 1985) into the *zur* mutant 1430nk and the wild-type strain 8004 by the triparental conjugation as described by Turner and associates (1985) using

pRK2073 (Leong et al. 1982) as the helper plasmid. Transconjugants were screened on the rich medium NYG (Daniels et al. 1984a) supplemented with appropriate antibiotics. The obtained reporter strains were named 1430nk(pGUS*hrpA*), 1430nk(pGUS*hrpB*), 1430nk(pGUS*hrpC*), and so on. In *Xanthomonas* spp., the expression of *hrp* genes, including the regulators *hrpG* and *hrpX*, has been shown to be induced in nutrient-deficient media but repressed in nutrient-rich media (Tang et al. 2006; Wengelnik et al. 1996a). Therefore, we measured the GUS activities of the obtained reporter strains in the minimal medium MMX (Daniels et al. 1984b). The results showed that the GUS activity of each of these *hrp-gusA* reporter plasmids, except pGUS*hrpG*, in the *zur* mutation background was significantly (*t* test, *P* = 0.01) lower than that in the wild-type background (Table 2), indicating that the transcription of all six *hrp* operons (*hrpA* to *hrpF*) and the *hrp* regulator *hrpX* is positively regulated by Zur in *X. campestris* pv. *campestris*. The GUS activities of the *hrpG*-promoter-*gusA* transcriptional fusion reporter plasmid in the *zur*-mutation background and the wild-type background (i.e., strains 1430nk(pGUS*hrpG*) and 8004(pGUS*hrpG*)) are almost identical (Table 2), suggesting that Zur is not required for the transcription of *hrpG*.

HrpX activates the expression of *hrpA* to *hrpF* operons in *X. campestris* pv. *campestris*.

As described above, the organization of the *hrpA* to *hrpF* operons in *X. campestris* pvs. *vesicatoria* and *campestris* is similar (Büttner et al. 2003) and it has been demonstrated that, in *X. campestris* pv. *vesicatoria*, HrpG activates the expression of the *hrpA* operon and *hrpX*, and HrpX then activates the expression of *hrpB* to *hrpF* operons but not *hrpA* (Wengelnik and Bonas 1996; Wengelnik et al. 1996b). To determine the regulatory relationship among the *hrp* genes in *X. campestris* pv. *campestris*, the reporter plasmids pGUS*hrpA*, pGUS*hrpB*, pGUS*hrpC*, pGUS*hrpD*, pGUS*hrpE*, pGUS*hrpF*, pGUS*hrpG*, and pGUS*hrpX* (Table 1) were introduced from the *E. coli*

Table 1. Bacterial strains and plasmids used in this work

Strains or plasmids	Relevant characteristics ^z	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> Δ (<i>lac-proAB</i>)/F' [<i>traD36</i> , <i>lacF</i> , <i>lacZAM15</i>]	Yanisch-Perron et al. 1985
<i>Xanthomonas campestris</i> pv. <i>campestris</i> 8004	Wild type, Rif ^r	Daniels et al. 1984a
1430nk	As 8004, but <i>zur</i> ::pK18mob, Rif ^r , Kan ^r	Tang et al. 2005
C1430nk	Complemented strain of 1430nk, Rif ^r , Kan ^r , Tc ^r	Tang et al. 2005
8004G	8004 <i>ΔhrpG</i> , Rif ^r , Kan ^r	Wei et al. 2007
8004X	8004 <i>ΔhrpX</i> , Rif ^r , Kan ^r	Wei et al. 2007
<i>hrcV</i> mutant	As 8004, but the coding region of <i>hrcV</i> (<i>XC3013</i>) has been deleted, Rif ^r	Laboratory collection
1430nk(pR3G)	1430nk harboring pR3G, Rif ^r , Kan ^r , Tc ^r	This work
8004(pR3G)	8004 harboring pR3G, Rif ^r , Kan ^r , Tc ^r	This work
1430nk(pR3X)	1430nk harboring pR3X, Rif ^r , Kan ^r , Tc ^r	This work
8004(pR3X)	8004 harboring pR3X, Rif ^r , Kan ^r , Tc ^r	This work
8004G(pR3X)	8004G harboring pR3X, Rif ^r , Kan ^r , Tc ^r	This work
Plasmids		
pLAFR6	Broad host range cloning vector, Tc ^r	Huynh et al. 1989
pLAFR3	Broad host range cloning vector, Tc ^r	Huynh et al. 1989
pRK2073	Helper plasmid, Tra ⁺ , Mob ⁺ , ColE1, Spc ^r	Leong et al. 1982
pXC1430	pLAFR6 containing an 833-bp fragment including <i>zur</i> gene, Tc ^r	Tang et al. 2005
pR3X	pLAFR3 containing a 1,523-bp fragment including promoterless <i>hrpX</i> gene, Tc ^r	This work
pR3G	pLAFR3 containing an 899-bp fragment including promoterless <i>hrpG</i> gene, Tc ^r	This work
pGUS <i>hrpA</i>	pLAFR6 containing an <i>hrpA</i> operon promoter- <i>gusA</i> fusion fragment, Tc ^r	Laboratory collection
pGUS <i>hrpB</i>	pLAFR6 containing an <i>hrpB</i> operon promoter- <i>gusA</i> fusion fragment, Tc ^r	Laboratory collection
pGUS <i>hrpC</i>	pLAFR6 containing an <i>hrpC</i> operon promoter- <i>gusA</i> fusion fragment, Tc ^r	Laboratory collection
pGUS <i>hrpD</i>	pLAFR6 containing an <i>hrpD</i> operon promoter- <i>gusA</i> fusion fragment, Tc ^r	Laboratory collection
pGUS <i>hrpE</i>	pLAFR6 containing an <i>hrpE</i> operon promoter- <i>gusA</i> fusion fragment, Tc ^r	Laboratory collection
pGUS <i>hrpF</i>	pLAFR6 containing an <i>hrpF</i> operon promoter- <i>gusA</i> fusion fragment, Tc ^r	Laboratory collection
pGUS <i>hrpG</i>	pLAFR6 containing an <i>hrpG</i> promoter- <i>gusA</i> fusion fragment, Tc ^r	Laboratory collection
pGUS <i>hrpX</i>	pLAFR6 containing an <i>hrpX</i> promoter- <i>gusA</i> fusion fragment, Tc ^r	Laboratory collection

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

JM109 into the *hrpG*-mutant strain 8004G, *hrpX*-mutant strain 8004X, and the wild-type strain 8004 by triparental conjugation. The GUS activities of the resulting reporter strains were measured after being grown in the minimal medium MMX. The results showed that the GUS activity of each of these *hrp-gusA* reporter plasmids in the *hrpG* or *hrpX* mutation background was significantly (*t* test, $P = 0.01$) lower than that in the wild-type background (Table 2), indicating that, in *X. campestris* pv. *campestris*, HrpX regulates the expression of all the six *hrp* operons (*hrpA* to *hrpF*). The reporter plasmids pGUS *hrpG* and pGUSHrpX were also introduced into the *hrpX* mutant and *hrpG* mutant, respectively. The GUS activity produced by the reporter plasmid pGUSHrpX in the *hrpG* mutant was significantly lower than that in the wild type (Table 2), denoting that the expression of *hrpX* is positively regulated by HrpG in *X. campestris* pv. *campestris*.

In *X. campestris* pv. *vesicatoria*, *hrpA* is activated by HrpG but not HrpX (Wengelnik and Bonas 1996). However, the data shown in Table 2 demonstrate that both HrpX and HrpG are required for the activation of *hrpA* in *X. campestris* pv. *campestris*.

Because the expression of *hrpX* is activated by HrpG, it is possible that HrpG regulates *hrpA* via *hrpX*. To clarify this possibility, the low-copy-number vector pLAFR3 with an *E. coli lac* promoter flanking the multiple cloning sites (Huynh et al. 1989), which expresses constitutively in *X. campestris* (Wengelnik et al. 1996a), was used to construct the recombinant plasmid pR3X (Table 1), in which the promoterless *hrpX* of *X. campestris* pv. *campestris* with its authentic RBS was cloned into pLAFR3 in an orientation that allowed the *hrpX* to be driven by the *lac* promoter (details below). The plasmid pR3X constitutively expressing *hrpX* was then introduced into the *hrpG* mutant 8004G and the RNA level of *hrpA* in the resulting strain 8004G(pR3X) was assessed by semiquantitative reverse-transcription polymerase chain reaction (RT-PCR). The results showed that the *hrpA* RNA level in 8004G(pR3X) was similar to that in the wild-type strain (Fig. 2A), suggesting that HrpG activates the expression of *hrpA* through *hrpX* in *X. campestris* pv. *campestris*. Taken together, the above results indicate that, in contrast to the situation in *X. campestris* pv. *vesicatoria*, HrpG does not control *hrpA* directly in *X. campestris* pv. *campestris*.

Fig. 1. Hypersensitive response (HR) symptoms induced in pepper leaves (*Capsicum annuum* cv. ECW-10R) by *Xanthomonas campestris* pv. *campestris* strains. The plants were inoculated by infiltrating approximately 5 μ l of bacterial suspension (1×10^8 CFU/ml) in 10 mM sodium phosphate buffer (5.8 mM Na_2HPO_4 and 4.2 mM NaH_2PO_4 , pH 7.0) into the pepper leaves using a blunt-end plastic syringe. The inoculated plants were maintained in a greenhouse with a 12-h day and night cycle with illumination by fluorescent lamps and a constant temperature of 28°C. HR symptoms were observed and photographed at 8 and 16 h post inoculation, respectively. At least three plants were inoculated in each experiment and each experiment was repeated twice. The results presented are from a representative experiment and similar results were obtained in all other independent experiments; 1, wild-type strain 8004; 2, *zur*-mutant 1430nk; 3, complemented strain C1430nk; 4, strain 1430nk(pR3X); 5, strain 1430nk(pR3G); and 6, type III secretion system-deficient strain (*hrcV* mutant; negative control).

Table 2. β -Glucuronidase (GUS) activities of the *hrp* promoter-*gusA* reporters in different genetic backgrounds grown in the minimal medium MMX

Reporter plasmid	GUS activities (U) in ^a			
	8004 (wild type)	1430nk (<i>zur</i> mutant)	8004G (<i>hrpG</i> mutant)	8004X (<i>hrpX</i> mutant)
pGUSHrpA	1.11 $\pm$ 0.27 A	0.36 $\pm$ 0.11 B	0.14 $\pm$ 0.03 C	0.12 $\pm$ 0.04 C
pGUSHrpB	1.99 $\pm$ 0.24 A	0.32 $\pm$ 0.06 B	0.10 $\pm$ 0.02 C	0.08 $\pm$ 0.01 C
pGUSHrpC	1.91 $\pm$ 0.17 A	0.37 $\pm$ 0.02 B	0.11 $\pm$ 0.02 C	0.13 $\pm$ 0.03 C
pGUSHrpD	1.69 $\pm$ 0.17 A	0.25 $\pm$ 0.04 B	0.08 $\pm$ 0.01 C	0.12 $\pm$ 0.03 C
pGUSHrpE	1.56 $\pm$ 0.36 A	0.42 $\pm$ 0.09 B	0.18 $\pm$ 0.02 C	0.17 $\pm$ 0.02 C
pGUSHrpF	1.33 $\pm$ 0.15 A	0.46 $\pm$ 0.04 B	0.10 $\pm$ 0.01 C	0.10 $\pm$ 0.01 C
pGUSHrpG	0.99 $\pm$ 0.03 A	0.91 $\pm$ 0.06 A	ND	1.01 $\pm$ 0.04 A
pGUSHrpX	0.92 $\pm$ 0.04 A	0.26 $\pm$ 0.06 B	0.09 $\pm$ 0.01 C	ND

^a GUS activities were determined after growth of *Xanthomonas campestris* pv. *campestris* strains in the minimal medium MMX for 48 h. Data are the mean $\pm$ standard deviation of triplicate measurements. The different letters in each horizontal data column indicate significant differences at $P = 0.01$. The experiment was repeated twice and similar results were obtained. ND = not done.

To get some clues about the differentiation of the *hrpA* regulation between *X. campestris* pv. *campestris* and *X. campestris* pv. *vesicatoria*, we compared their intergenic region (IGR) DNA sequences between *hrpB8* and *hrcC* (*hrpA* operon). The result showed that i) the lengths of the two IGR are different, 176 and 81 bp in *X. campestris* pv. *campestris* and *X. campestris* pv. *vesicatoria*, respectively; and ii) their first 26 nucleotide sequences upstream of *hrcC* start codons are highly conserved (88% identity) but the remainders show low sequence similarity (Fig. 3). These sequence variations may relate to the *hrpA* regulation diversity between *X. campestris* pv. *campestris* and *X. campestris* pv. *vesicatoria* (discussed below).

Zur activates the expression of *hrpA* to *hrpF* operons through HrpX.

It has been demonstrated that, in *X. campestris* pv. *vesicatoria*, HrpX regulates the transcription of *hrp* genes by directly

binding to the plant-inducible promoter (PIP) box present in the promoters of *hrp* operons (Koebnik et al. 2006). It is possible that, in *X. campestris* pv. *campestris*, Zur does not regulate directly the expression of the six *hrp* operons but, instead, via HrpX that then controls directly the expression of the *hrp* genes with the PIP box. If so, constitutively expressing *hrpX* but not *hrpG* would bypass the requirement of *zur* for the expression of *hrp* genes. To verify this hypothesis, a recombinant plasmid named pR3G (Table 1), which constitutively expresses *hrpG*, was also constructed by cloning the promoterless *hrpG* of *X. campestris* pv. *campestris* into pLAFR3 similarly to the construction of the recombinant plasmid pR3X above. The plasmids pR3X and pR3G were introduced into the *zur* mutant 1430nk and the wild-type strain 8004 by triparental conjugation, respectively. The obtained transconjugant strains were named 1430nk(pR3X), 8004(pR3X), 1430nk(pR3G), and 8004(pR3G) (Table 1). The virulence and HR of these strains were determined in the

Fig. 2. Detection of the mRNA level of *hrp* genes in *Xanthomonas campestris* pv. *campestris* strains by semiquantitative reverse-transcription polymerase chain reaction (PCR). RNA was isolated from cultures of the *X. campestris* pv. *campestris* strains grown to an optical density at 600 nm of 1.0 in the MMX medium (Daniels et al. 1984b). The same amount of RNA from the testing strains in the same comparable group was used to convert to cDNA using a TaKaRa RNA PCR Kit (AMV; ver. 3.0; TaKaRa). PCR amplifications with *Taq* polymerase were performed by using the obtained cDNAs as templates and the related primer sects. The obtained PCR products were analyzed in 1.2% agarose gels. The 16S rRNA gene of *X. campestris* pv. *campestris* was used as the internal control to verify the absence of significant variation at cDNA level in the samples. The experiment was repeated twice and similar results were obtained.

host plant Chinese radish (*Raphanus sativus* L. var. *radiculus* Pers.) and the nonhost plant pepper ECW-10R. As shown in Figure 1, the strain 1430nk(pR3X) induced a wild-type HR, while the strain 1430nk(pR3G) induced a delayed and weakened HR similar to that caused by the *zur* mutant 1430nk, indicating that constitutively expressing *hrpX* but not *hrpG* in the *zur* mutant could completely restore the HR-eliciting capability of the mutant. This demonstrates that *X. campestris* pv. *campestris* Zur affects the expression of the *hrp* cluster of genes via HrpX. The mean lesion length caused by 1430nk(pR3X) was far shorter than that produced by the wild type, although it was significantly (*t* test, *P* = 0.01) longer than that produced by the *zur* mutant (Table 3), suggesting that constitutively expressing *hrpX* in the *zur* mutant could only partially restore the virulence of the *zur* mutant. Constitutive expression of *hrpG* in the *zur* mutant strain and the wild-type strain did not alter significantly the virulence of the strains (Table 3).

To investigate whether the HR restoration of the *zur* mutant 1430nk by constitutively expressing *hrpX* accompanies the restoration of the mRNA level of the relative *hrp* genes, we assayed the mRNA level of *hrcC* (XC3003) (*hrpA* operon), *hrpB8* (XC3004) (*hrpB* operon), *hrcU* (XC3012) (*hrpC* operon), *hrcR* (XC3016) (*hrpD* operon), *hrpE* (XC3021) (*hrpE* operon), *hrpF* (XC3025) (*hrpF* operon), and *hrpX* (XC3076) in the strains 1430nk(pR3X) and 1430nk(pR3G) by semiquantitative RT-PCR. As shown in Figure 2B, i) the mRNA levels of all the tested *hrp* genes in strains 8004, 8004(pR3X), and 8004(pR3G) were similar, indicating that constitutive expression of *hrpG* or *hrpX* in the wild-type background did not increase the expression of downstream genes significantly; and ii) the mRNA levels of all the tested *hrp* genes in the strain 1430nk(pR3X) were similar to those in the strain 8004(pR3X) but significantly higher than those in the strain 1430nk(pR3G), suggesting that *zur* does not regulate the transcription of *hrpG*. In addition, the recombinant plasmid pR3G could fully restore the virulence and HR of the *hrpG* mutant to wild type (data not shown), indicating that the pR3G construct expresses a functional HrpG protein. These observations further demonstrate that Zur positively regulates the transcription of *hrpX* but not *hrpG* and the former then controls the expression of the *hrpA* to *hrpF* operons in *X. campestris* pv. *campestris*.

The effects of Zur on HR induction, extracellular polysaccharide production, and zinc homeostasis are probably through different regulatory pathways.

Our previous work has shown that inactivation of *zur* also resulted in a reduction of extracellular polysaccharide (EPS) of *X. campestris* pv. *campestris* (Tang et al. 2005). It has been well established that EPS is an important virulence factor of *X. campestris* pv. *campestris* (Chan and Goodwin 1999). To verify whether or not constitutively expressing *hrpX* in the *zur* mutant 1430nk can restore the EPS production of the mutant, we measured the EPS production of 1430nk(pR3X). The result showed that the EPS yield of 1430nk(pR3X) was almost the

same as that of the *zur* mutant 1430nk (Table 3), indicating that constitutively expressing *hrpX* cannot restore the EPS production of the *zur* mutant. This result fits in with the above observation that constitutively expressing *hrpX* could only partially restore the virulence of the *zur* mutant. However, in addition to EPS, whether there are other virulence factors affected by *zur* in *X. campestris* pv. *campestris* remains to be investigated. Nevertheless, these results demonstrate that the regulation of EPS production by *zur* is not mediated by *hrpX* in *X. campestris* pv. *campestris*. In addition, we compared the Zn²⁺ tolerance of strain 1430nk(pR3X) and the *zur* mutant 1430nk, and found that constitutively expressing *hrpX* did not alter the Zn²⁺ tolerance of the *zur* mutant (data not shown). Overall, these results reveal that the HR induction, EPS production, and Zn²⁺ homeostasis controlled by Zur is achieved through different regulatory cascades in *X. campestris* pv. *campestris*.

Zur does not bind to the *hrpX* promoter in vitro under conditions contented for the binding of Zur to the promoter of genes involved in zinc homeostasis.

It has been demonstrated that Zur can regulate gene expression directly or indirectly, and the mechanism of the direct regulation is through the binding of Zur to the promoter region of target genes (Huang et al. 2008; Outten and O'Halloran 2001; Patzer and Hantke 2000). To determine whether Zur regulates the expression of *hrpX* directly in *X. campestris* pv. *campestris*, we employed an electrophoretic mobility shift assay (EMSA) to study whether Zur binds to the *hrpX* promoter in vitro. An His₆-tagged Zur protein of *X. campestris* pv. *campestris* was purified as described previously (Huang et al.

Table 3. Effects of constitutive expression of *hrpX* and *hrpG* on the virulence and extracellular polysaccharide (EPS) production of *Xanthomonas campestris* pv. *campestris* strains^z

Strains	Lesion length (mm)	EPS yield (g/liter)
8004	12.2 ± 3.4 A	13.7 ± 0.2 A
1430nk	1.5 ± 1.1 B	4.5 ± 0.4 B
1430nk(pXC1430)	11.6 ± 2.8 A	14.9 ± 0.4 A
8004(pR3G)	11.5 ± 2.9 A	12.9 ± 0.6 A
1430nk(pR3G)	1.2 ± 0.7 B	4.4 ± 0.3 B
8004(pR3X)	11.8 ± 2.4 A	13.2 ± 0.8 A
1430nk(pR3X)	4.6 ± 1.2 C	4.9 ± 0.6 B

^z Virulence and EPS production were assayed by the methods described previously (Tang et al. 2005). Virulence of the *X. campestris* pv. *campestris* strains was tested on the host plant Chinese radish (*Raphanus sativus* L. var. *radiculus* Pers.) by leaf-clipping. The concentration of bacterial inocula was optical density at 600 nm = 0.1. Lesion length was measured 10 days post inoculation. Values are the means ± standard deviations from three repeats, each with 60 leaves. EPS production of the strains was measured after grown in NYG medium (Daniels et al. 1984a) containing 4% glucose at 28°C with shaking at 200 rpm for 5 days. Data are the means ± standard deviations of triplicate measurements. The same experiment was repeated three times, and similar results were obtained. Different letters in each data column indicate significant differences at *P* = 0.01.

Fig. 3. Alignment of the nucleotide sequence of the intergenic regions (IGR) between *hrpB8* and *hrcC* of *Xanthomonas campestris* pv. *campestris* and *X. campestris* pv. *vesicatoria*. The two sequences were from the sequence databases of National Center for Biotechnology Information (GenBank accession numbers CP000050 for *X. campestris* pv. *campestris* 8004 genome and AM039952 for *X. campestris* pv. *vesicatoria* 85-10 genome). The boxed area denotes the conserved region and the arrow indicates the start codon and the orientation of *hrcC* open reading frame.

2008). A 230-bp DNA fragment containing the *hrpX* promoter, named P_{hrpX} , was amplified by PCR using the total DNA of the *X. campestris* pv. *campestris* wild-type strain 8004 as template and the primer set *PhrpX*-F/*PhrpX*-R (Supplementary Table S1). The binding ability of the purified *X. campestris* pv. *campestris* His₆-Zur to the amplified *hrpX* promoter DNA fragment P_{hrpX} was then tested by EMSA as described previously (Huang et al. 2008). The result showed that the *X.*

campestris pv. *campestris* His₆-Zur did not bind to the DNA fragment P_{hrpX} even at a very high level (3,200 nM) of His₆-Zur (Fig. 4), although it bound with a high affinity to P_{3788} (Fig. 4), a 275-bp DNA fragment containing the promoter of *XC3788*, a gene that has been demonstrated to be directly regulated by Zur (Huang et al. 2008). This result suggests that Zur may regulate *hrpX* indirectly in *X. campestris* pv. *campestris*, or the binding of Zur with the *hrpX* promoter needs other conditions which are present in vivo.

DISCUSSION

This study has demonstrated that the *X. campestris* pv. *campestris* Zur is implicated in HR and positively regulates the transcription of *hrp* cluster and *hrpX* but not *hrpG*. The data presented in this article further demonstrated that Zur controls the transcription of *hrpA* to *hrpF* operons via HrpX. Constitutive expression of *hrpG* could not restore the transcription level of the *hrp* cluster in the *zur* mutant to wild type, suggesting that Zur does not act as a regulator for the transcription of *hrpG*. However, whether Zur acts as a post-transcriptional regulator of *hrpG* or a cofactor required for HrpG to fully activate *hrpX* and downstream *hrp* operons remains to be further investigated.

Recently, it has been shown that, similar to the *X. campestris* pv. *campestris* Zur, the *X. oryzae* pv. *oryzae* Zur is also required for Zn²⁺ homeostasis, virulence, and EPS production (Yang et al. 2007). Interestingly, from a whole-genome microarray hybridization analysis, He and co-workers (2007a) found that the Zur of *X. campestris* pv. *campestris* XC1 does not regulate the expression of *hrp* genes. This observation reflects that different *X. campestris* pv. *campestris* strains may have evolved genetic diversity of the Zur regulatory functions on pathogenicity and other physiological traits. Indeed, it has been shown that, in the strain XC1, Zur is not required for EPS production, although its involvement in HR production is unknown (He et al. 2007a).

The *hrp* cluster (*hrpA* to *hrpF* operons) and the *hrp* key regulators *hrpG* and *hrpX* are highly conserved among different *Xanthomonas* spp. and pathovars; therefore, it has long

Fig. 4. Determination of Zur-binding to promoter regions by electrophoretic mobility shift assay. His₆-Zur protein was incubated with 3 pmol of ³²P-end-labeled DNA fragment containing the promoter region of *XC3788* (P_{3788}) or *hrpX* (P_{hrpX}) in 20 μ l of binding buffer (20 mM Tris-HCl [pH 8], 50 mM KCl, 1 mM dithiothreitol, 5% glycerol, bovine serum albumin at 0.1 mg/ml, and sheared salmon sperm DNA at 5 μ g/ml) at 28°C for 15 min, and then analyzed by 4% polyacrylamide gel electrophoresis. The experiment was repeated twice and similar results were obtained.

Fig. 5. Working model of *hrp* gene regulation in *Xanthomonas campestris* pv. *campestris*. HrpG and Zur regulate positively the expression of *hrpX* and HrpX then activates the *hrp* cluster of genes (*hrpA* to *hrpF* operons) and *hpaR*. *rsmA* and *colR/S* are not regulated by Zur, HrpG, or HrpX. *RsmA* positively controls the expression of the *hrp* cluster (*hrpA* to *hrpF* operons) while *ColR/S* is required for the expression of *hrpC* and *hrpE* operons only.

been considered that the regulation of the *hrp* cluster is similar among different *Xanthomonas* bacteria. In this work, we have validated genetically that, in *X. campestris* pv. *campestris*, similar to the HrpG of *X. campestris* pv. *vesicatoria*, HrpG regulates positively the expression of *hrpX*; however, HrpX controls the expression of all of the six *hrp* operons (i.e., *hrpA* to *hrpF*), differing from the finding in *X. campestris* pv. *vesicatoria* in which HrpX controls the expression of only five of the six *hrp* operons (i.e., *hrpB* to *hrpF* but not *hrpA*) (Wengelnik and Bonas 1996). The upstream sequence of the *HrcC* open reading frame (ORF) (*hrpA* operon) of *X. campestris* pv. *campestris* differs from that of *X. campestris* pv. *vesicatoria* (Fig. 3), suggesting that it may not be recognized by HrpG. Whether HrpX activates directly the *X. campestris* pv. *campestris* *hrpA* is not clear, because no typical or imperfect PIP box motif (Cunnac et al. 2004; Koebnik et al. 2006) is present in the *hrpA* promoter region (Fig. 3). A number of genes without a PIP box, including *hrpE* operon, have been described as being expressed in an HrpX-dependent manner and possible indirectly regulatory models have been proposed (Koebnik et al. 2006).

Interestingly, a BlastN result showed that the sequences of the IGR between *hrpB8* and *hrcC* (the upstream sequence of *hrpA* ORF) in *Xanthomonas* spp. fall into two groups: group 1 is a long IGR (181 bp), which is present in all of the sequenced *X. campestris* pv. *campestris* 8004, ATCC33913, and B100 (almost identical among the three strains); and group 2 is a short IGR (81 bp), which exists in all of the sequenced strains of *X. campestris* pv. *vesicatoria*, *X. axonopodis* pv. *citri*, *X. fuscans* subsp. *fuscans*, *X. axonopodis* pv. *glycines*, and *X. oryzae* pv. *oryzae* (approximately 90% identity among these strains). Recently, Seo and associates (2008) found that, except for *hrpA1* and *hrpE*, other *hrp* genes of *X. oryzae* pv. *oryzicola* are not induced in the minimal medium XOM2, a condition in which most of the *hrp* genes of *X. oryzae* pv. *oryzae* are induced. Therefore, they concluded that the regulation of the *hrp* genes between *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* is different (Seo et al. 2008). To further investigate the regulatory relationship among *hrpA*, *hrpG*, and *hrpX* of the above xanthomonads will help us to fully understand the *hrp* regulatory mechanisms.

In *Xanthomonas* spp., the *hrpA* to *hrpD* operons contain nine *hrc* (*hrp*-conserved) genes that encode proteins that presumably constitute the core components of the T3SS apparatus in the inner and outer membrane of bacterial cells, and *hrpE* and *hrpF* encode pilin and translocon proteins, respectively, which are also important for T3SS (Büttner et al. 2003; Gürlbeck et al. 2006). The significant reduction in expression of these genes in the *zur* mutant may affect the T3SS assembly, resulting in a delayed and weakened HR.

Because a mutation in *hrpG* does not affect zinc homeostasis of *X. campestris* pv. *campestris* (data not shown), it is logical to assume that the expression of *zur* is not under the control of HrpG. A putative transcriptional regulatory gene, named *trh*, has been demonstrated to be involved in the expression of *hrpG* of *X. oryzae* pv. *oryzae*, although whether the regulation is direct or indirect is unknown (Tsuge et al. 2006). Recently, we have identified several additional regulatory genes, *hpaR*, *rsmA_{Xcc}*, *colR_{XC1049}*, and *colS_{XC1050}*, which are involved in HR and pathogenicity of *X. campestris* pv. *campestris* (Chao et al. 2008; Wei et al. 2007; Zhang et al. 2008). *hpaR* is a marR family transcriptional regulator and its expression is positively controlled by HrpG and HrpX, and it does not regulate the expression of *hrpA* to *hrpF* operons (Wei et al. 2007). *RsmA_{Xcc}* is a putative RNA-binding protein involved in the control of various cellular processes and positively regulates the expression of *hrpA* to *hrpF* operons but not *hrpG* and *hrpX* (Chao et

al. 2008). *colR_{XC1049}* and *colS_{XC1050}* encode a putative two-component signal transduction system implicated in virulence, HR, and tolerance to various stresses, and positively regulates the expression of *hrpC* and *hrpE* operons but not other *hrp* genes; and the expression of *colR_{XC1049}* and *colS_{XC1050}* is not controlled by HrpG and HrpX (Zhang et al. 2008). To the best of our knowledge, concerning *hrp* regulation only, the *X. campestris* pv. *campestris* Zur represents a novel key regulator that acts upstream of the key regulator HrpX but is not regulated by HrpG (Fig. 5).

This work has also shown that the effects of Zur on HR induction, EPS production, and zinc homeostasis are probably through different regulatory pathways in *X. campestris* pv. *campestris*. Promoter-*gusA* reporter analysis revealed that the *X. campestris* pv. *campestris* Zur may not regulate the expression of the EPS production-associated genes *clp*, *gumB*, *opsX*, and *rpfC* (Tang et al. 2005). Recently, we have demonstrated that Zur functions as a repressor and an activator of putative zinc homeostasis genes via recognizing two distinct sequences within its target promoters in *X. campestris* pv. *campestris* (Huang et al. 2008). Bacterial Zur is a member of the Fur family of transcriptional regulators. Ellermeier and Schlauch (2008) have demonstrated that the Fur of the animal pathogen *Salmonella enterica* serovar *typhimurium* regulates expression of the *Salmonella* pathogenicity island 1 (SPI1) T3SS through the AraC-like transcriptional activator HilD, a key regulator of *Salmonella* T3SS, which can activate *hilaA* that encodes the direct regulator of the SPI1 structural genes. They have also shown that the Fur does not directly regulate the transcription of *hilD*, although the mechanism by which the Fur regulates *hilD* is still unclear (Ellermeier and Schlauch 2008). To further investigate the mechanism by which Zur controls the expression of *hrpX* and the production of EPS will facilitate our understanding of the regulatory mechanisms of the Fur regulator family.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions.

The bacterial strains and plasmids used in this work are listed in Table 1. *E. coli* strains were grown in L medium (Miller 1972) at 37°C. *X. campestris* pv. *campestris* strains were grown in NYG medium (Daniels et al. 1984a) or MMX medium (Daniels et al. 1984b) at 28°C. Antibiotics were used at the following final concentrations: ampicillin, 100 µg/ml; chloramphenicol, 100 µg/ml; kanamycin, 25 µg/ml; rifampicin, 50 µg/ml; and tetracycline, 15 µg/ml for *E. coli* and 5 µg/ml for *X. campestris* pv. *campestris*.

DNA manipulation.

DNA manipulation was performed following the procedures described by Sambrook and associates (1989). The conjugation between the *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, China).

Construction of pR3X and pR3G.

To construct *hrpX* and *hrpG* constitutive expression plasmids, 1,523- and 899-bp DNA fragments, including promoterless *hrpX* and *hrpG* genes, respectively, with RBS were amplified by PCR using the total DNA of the wild-type strain 8004 as template and the primer sets *hrpXPF/hrpXPR* and *hrpGPF/hrpGPR*. After purification using the QIAquick PCR purification kit (Qiagen, Hilden, Germany), the two fragments were confirmed by sequencing and cloned into the broad-host-

range vector pLAFR3 under the control of *lac* promoter to generate the recombinant plasmids pR3X and pR3G (Table 1).

Semiquantitative RT-PCR analysis.

To carry out semiquantitative RT-PCR analysis, RNA was isolated from cultures of the *X. campestris* pv. *campestris* strains grown to an optical density at 600 nm (OD_{600}) of 1.0 in the minimal medium MMX. The same amount of RNA from different strains was used to convert to cDNA using a TaKaRa RNA PCR Kit (AMV; ver. 3.0; TaKaRa Biotechnology Co., Ltd. Dalian, China). The cDNAs were used as templates for PCR amplification with *Taq* polymerase. PCR was performed with a cycler using the following cycle parameters: 30 cycles of 94°C for 15 s, 60°C for 15 s, and 72°C for 15 s. The amplified products were analyzed in 1.2% agarose gels. The 16S rRNA gene of *X. campestris* pv. *campestris* was used as the internal control to verify the absence of significant variation at cDNA level in the samples.

GUS activity assay.

X. campestris pv. *campestris* strains were cultured in the minimal medium MMX for 48 h. GUS activities were determined by measurement of the OD_{415} , using *p*-nitrophenyl β -D-glucuronide as substrate, as described by Jefferson and associates (1986).

Measurement of EPS production.

The EPS production was measured as described by Wilson and associates (1998) with a minor modification. Overnight culture (OD_{600} of approximately 1.0) of each *X. campestris* pv. *campestris* strain (2 ml) was used to inoculate 100 ml of NYG medium containing 4% glucose in a 500-ml flask and kept at 28°C with shaking at 200 rpm for 4 days. The EPS was precipitated from the culture supernatant by addition of 3 volumes of 95% ethanol. The pelleted EPS was washed with 70% ethanol, air dried, and weighed. Three flasks were inoculated in each experiment and each experiment was repeated three times.

Determination of HR.

The HR was tested on pepper cv. ECW-10R (*C. annuum* cv. ECW-10R), which is one of the nonhosts commonly used to test the HR of *X. campestris* pv. *campestris* (Castañeda et al. 2005; Newman et al. 2001). The plants were inoculated by infiltrating approximately 5 μ l of bacterial suspension (1×10^8 CFU/ml) in 10 mM sodium phosphate buffer (5.8 mM Na_2HPO_4 and 4.2 mM NaH_2PO_4 , pH 7.0) into the pepper leaves by a blunt-end plastic syringe. The inoculated plants were maintained in a greenhouse with a 12-h day and night cycle, illumination by fluorescent lamps, and a constant temperature of 28°C and were observed at 8, 16, 24, 36, and 48 h after inoculation. At least three plants were inoculated in each experiment and each experiment was repeated at least twice.

Virulence assay.

The virulence of the *X. campestris* pv. *campestris* strains was tested on potted Chinese radish (*R. sativus* L. var. *radiculus* Pers.) grown in a greenhouse with a 12-h day and night cycle and illumination by fluorescent lamps at 25 to 28°C. Seedlings with four fully expanded leaves were used for inoculation. Bacterial cells were grown in NYG medium at 28°C with shaking at 200 rpm for 15 h (at the exponential phase of growth). The concentration of bacterial inocula was adjusted to OD_{600} of 0.1. Two leaves per plant were inoculated by the leaf-clipping method (Dow et al. 2003). Sixty leaves were inoculated for each independent experiment. After maintenance in 100% humidity for 24 h, the inoculated plants were main-

tained in the growth conditions described above. Lesion length was measured 10 days post inoculation. The same experiment was repeated three times.

EMSA.

EMSA was performed following the procedure described by Gaballa and Helmann (1998). DNA fragments containing the promoter regions of *XC3788* (P_{3788}) or *hrpX* (P_{hrpX}) were amplified by PCR using the total DNA of the wild-type strain 8004 as template and the primer sets P_{3788} -F/ P_{3788} -R and P_{hrpX} -F/ P_{hrpX} -R. After purification using the QIAquick PCR purification kit (Qiagen), the obtained PCR products were labeled with [γ - 32 P]ATP using a 5' end-labeled kit (TaKaRa). Then, the His₆-Zur protein (Huang et al. 2008) was added into a 1.5-ml EP tube containing 20 μ l of binding buffer (20 mM Tris-HCl [pH 8], 50 mM KCl, 1 mM dithiothreitol, 5% glycerol, bovine serum albumin at 0.1 mg/ml, and sheared salmon sperm DNA at 5 μ g/ml) to final concentrations of 0, 32, 320, and 3200 nM, and 3 pmol of 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, Piscataway, NJ, U.S.A.).

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (30671142 and 30730004) and the '863' Program of the Ministry of Science and Technology of China (2006AA02Z175).

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