The \textit{xrvA} gene of \textit{Xanthomonas oryzae pv. oryzae}, encoding an H-NS-like protein, regulates virulence in rice

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\textit{Xanthomonas oryzae pv. oryzae} (\textit{Xoo}) causes bacterial blight disease in rice, one of the most serious rice diseases. The \textit{xrvA} gene from \textit{Xoo} strain 13751 encodes a protein containing a histone-like nucleoid-structuring protein (H-NS) domain. The expression of \textit{xrvA} in strain 13751 was enhanced in XOM2 minimal medium. Mutation of the \textit{xrvA} gene of strain 13751 led to a significant reduction in virulence in the host plant rice, a delayed hypersensitive response in the nonhost castor-oil plant, a decrease in extracellular polysaccharide and diffusible signal factor production, and an increase in intracellular glycogen accumulation. Northern hybridization analyses revealed that the virulence-associated genes \textit{hrpG}, \textit{hrpX}, \textit{rpfC}, \textit{rpfF}, \textit{rpfG} and \textit{gumB} were downregulated in the \textit{xrvA} mutant compared to the wild-type and complemented strains. Interestingly, increase of copy number of \textit{xrvA} in the wild-type strain 13751 resulted in a strain showing similar phenotypes as the \textit{xrvA} mutant and a reduction of the expression of \textit{gumB}, \textit{hrpX}, \textit{rpfC}, \textit{rpfF} and \textit{rpfG}. These findings indicate that the \textit{xrvA} gene, which is highly conserved in the sequenced strains of \textit{Xanthomonas}, encodes an important regulatory factor for the virulence of \textit{Xoo}.

INTRODUCTION

\textit{Xanthomonas oryzae pv. oryzae} (hereafter \textit{Xoo}), a member of the \textit{γ}-subdivision of the Gram-negative \textit{Proteobacteria}, is the causative agent of bacterial blight in rice (\textit{Oryza sativa} L.), the most serious bacterial disease of rice in many rice-growing areas worldwide. The pathogen invades rice leaves through hydathodes or wounds (Ou, 1985). After sufficient multiplication in the intercellular spaces of the underlying epiphyte, the bacteria enter the xylem and spread in the vascular system (Mew et al., 1993). Bacterial cells and extracellular polysaccharide (EPS) fill the xylem vessels within a few days (Nino-Liu et al., 2006).

A considerable effort is being made to identify genes involved in the pathogenesis of \textit{Xoo} to plants and to understand the roles of the gene products in the disease process. This work will undoubtedly be facilitated by the recent determination of the full genome sequence of \textit{Xoo} strains KACC10331, MAFF311018 and PXO99A (Lee et al., 2005; Ochiai, et al., 2005; Salzberg et al., 2008). It has already been established that the outcome of interactions of \textit{Xoo} with plants is determined by hypersensitive response and pathogenicity (\textit{hrp}) genes, which are required for pathogenicity in susceptible host plants and for the hypersensitive response (HR) in resistant and nonhost plants (Alfano & Collmer, 1997; Nino-Liu et al., 2006), and avirulence (\textit{avr}) genes that determine host specificity via gene-for-gene interactions (Bai et al., 2000; Lee et al., 2005; Nino-Liu et al., 2006). Other factors that contribute to the virulence of \textit{Xoo} include EPS, the type II general secretion system and its secreted proteins, and regulation involving genes within the \textit{rpf} cluster (Dharmapuri & Sonti, 1999; Dharmapuri et al., 2001; Nino-Liu et al., 2006; Tang et al., 1996; Chatterjee & Sonti, 2002; Jeong et al., 2008).

In xanthomonads, the \textit{hrp} gene cluster comprises six operons (\textit{hrpA} to \textit{hrpF}) and is positively regulated by HrpG and HrpX (Bonas et al., 1991; Wengelnik et al., 1996; Wengelnik & Bonas, 1996), repressed in nutrient-rich

A supplementary table of primers is available with the online version of this paper.

Abbreviations: DSF, diffusible signal factor; EPS, extracellular polysaccharide; H-NS, histone-like nucleoid-structuring protein; GUS, β-glucuronidase; HR, hypersensitive response; \textit{Xcc}, \textit{Xanthomonas campestris pv. campestris}; \textit{Xoo}, \textit{Xanthomonas oryzae pv. oryzae}.

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media, but induced in nutrient-limited media and inside the host (Arlat et al., 1991; Schulte & Bonas, 1992a, b; Tsuge et al., 2002). HrpG regulates the expression of hrpX, and HrpX then activates the expression of other hrp genes (Wengelnik et al., 1996; Wengelnik & Bonas, 1996). The highly conserved hrp genes named hrc encode the proteins of the apparatus of the type III secretion system and are critical for pathogenicity and the initiation of disease (Bogdanove et al., 1996; Alfano & Collmer, 1997; Lahaye & Bonas, 2001).

The rpf gene cluster (for regulation of pathogenicity factors) is involved in diverse regulatory actions contributing to virulence in different xanthomonads. Several of these rpf genes encode elements of a cell–cell communication mechanism which has been best studied in the crucifer pathogen Xanthomonas campestris pv. campestris (hereafter Xcc). The virulence of Xcc depends upon cell–cell signalling mediated by a diffusible signal factor (DSF) that has been characterized as cis-11-methyl-2-dodecenoic acid (Barber et al., 1997; Wang et al., 2004). The synthesis of the DSF in Xcc is fully dependent on RpfF and partially dependent on RpfB (Barber et al., 1997; Slater et al., 2000). The RpfC/RpfG two-component regulatory system is implicated in DSF perception and signal transduction. RpfG is an HD-GYP domain-containing protein that functions in cyclic di-GMP turnover in Xcc (Slater et al., 2000; Ryan et al., 2006).

The rpfB, rpfC, rpfF and rpfG genes of Xoo have been shown to affect virulence in rice, EPS production, xylanase production and motility (Tang et al., 1996; Chatterjee & Sonti, 2002; Jeong et al., 2008).

EPS synthesis in Xcc is directed by genes within the gum cluster, which contains 12 genes and has a major promoter upstream of the first gene, gumB (Katzen et al., 1996). Similarly, the Xoo gum cluster is composed of 14 ORFs that constitute an operon expressed from a promoter located upstream of gumB, but which also has internal promoters upstream of gumG, gumH and gumM, respectively (Lee et al., 2005; Yoon & Cho, 2007; Lee et al., 2008a).

The work described in this paper concerns the xrvA gene of Xoo, which encodes a protein containing an H-NS domain. H-NS and H-NS-like proteins are modular proteins associated with the bacterial nucleoid. These proteins have been identified in a number of bacteria, including Escherichia coli, Erwinia amylovora, Erwinia chrysanthemi, Proteus mirabilis, Salmonella typhimurium, Shigella flexneri and Vibrio cholerae, where they are known to act mainly as transcriptional repressors of a wide range of genes (reviewed by Tendend & Bertin, 2003; Dorman, 2004, 2007; Fang & Rimsky, 2008). The phenotypes of hns mutants are highly pleiotropic. For example, hns mutants of E. coli are able to use β-glucoside as a carbon source, are strongly susceptible to serine, mucoid, non-motile, and show increased resistance to low pH and to high osmolarity (reviewed by Tendend & Bertin, 2003). The H-NS proteins of E. coli, Er. amylovora, Er. chrysanthemi, Sal. typhimurium and Actinobacillus pleuropneumoniae have been shown to be involved in virulence (Müller et al., 2006; Hildebrand et al., 2006; Nasser et al., 2001; Harrison et al., 1994; Dalai et al., 2009). Here, we present genetic evidence to demonstrate that the xrvA gene of Xoo plays an important role in virulence and regulates the expression of a number of virulence genes.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** Table 1 lists the strains and plasmids used throughout this work. E. coli strains were grown in LB medium (Miller, 1972) at 37 °C. Xoo strains were grown in OB medium (Tang et al., 1996), containing (g l−1) polypeptone, 2; tryptone, 5; sucrose, 10; sodium glutamate, 1; L-methionine, 0.1; K2HPO4, 0.72; KH2PO4, 0.28; NH4Cl, 1; MgCl2, 1; FeCl2-EDTA, 1 p.p.m., or XOM2 medium (Tsuge et al., 2002) at 28 °C. Xcc strains were grown in NYGB medium (Daniels et al., 1984) at 28 °C. Antibiotics were used at the following final concentrations as required: streptomycin at 250 μg ml−1, kanamycin at 25 μg ml−1, ampicillin at 100 μg ml−1, rifampicin at 50 μg ml−1, and tetracycline at 15 μg ml−1 for E. coli and 5 μg ml−1 for Xoo.

**DNA manipulations and primer extension.** DNA manipulations were carried out as described by Sambrook & Russell (2001). The transcriptional start site of the xrvA gene was determined by primer extension analysis using the AMV reverse transcriptase Primer Extension System (Promega). The nonpolar mutant of xrvA in Xoo strain 13751 (Tang et al., 1996) was constructed by homologous suicide plasmid integration as described by Windgassen et al. (2000), using pK18MobGII as the vector (Katzen et al., 1999). The DNA sequence containing xrvA nucleotides 4–255 was amplified using the total DNA of strain 13751 as the template and the primer pair xrvANF/xrvANR, which was designed based on the xrvA sequence (GenBank accession no. X97866) (primer sequences are listed in Supplementary Table S1, available with the online version of this paper). The amplified DNA fragment was ligated to the plasmid vector pGEM-3Z(+) (Table 1) and confirmed by sequencing. The correct insert DNA was cloned into the suicide plasmid pK18MobGII to create the recombinant plasmid pK18MobGIIxrvA (Table 1). To ensure the creation of a nonpolar mutant, the 252 bp fragment was inserted such that the transcription orientation of the fragment was the same as that of the lac promoter in the vector. The plasmid pK18MobGIIxrvA was transferred from E. coli strain DH5α (Hanahan, 1983) into Xoo strain 13751 by triparental conjugation using pRK2073 (Leong et al., 1982) as the helper plasmid, as described by Turner et al. (1985). Transconjugants were screened on OA [OB with 1.5% (w/v) agar plates supplemented with streptomycin and kanamycin. The transconjugants obtained with a mutation in the xrvA gene were confirmed by PCR using the total DNAs of the transconjugants as the templates and the primer pair pK18F/Mu577R (Table S1) (pK18F is located in pK18MobGII, and Mu577R is located downstream of the 252 bp internal fragment of xrvA). The expected 843 bp PCR products were further confirmed by sequencing. One of the confirmed mutants, named GXN1280 (Table 1), was used for further study.

**Complementation of the xrvA mutant GXN1280.** In order to complement the xrvA mutant GXN1280, a 901 bp DNA fragment containing the entire xrvA gene (from 264 bp upstream of the start codon to 235 bp downstream of the stop codon) was amplified by PCR using the total DNA of the wild-type strain 13751 as the
Table 1. Bacterial strains and plasmids used in this work

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics*</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
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<tr>
<td>DH5α</td>
<td>recA φ80ΔlacZ ΔM15 Δ(lacZYA-argF)U169 endA1 recA1 deoR hsdR17 (rK mK ) phoA supE44 λ− thi-1 gyrA96 relA1</td>
<td>Hanahan (1983)</td>
</tr>
<tr>
<td><strong>Xoo</strong></td>
<td></td>
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<tr>
<td>13751</td>
<td>Laboratory wild-type, Chinese isolate, Sm'</td>
<td>Tang et al. (1996)</td>
</tr>
<tr>
<td>GXN1280</td>
<td>As 13751, but xrvA:: pK18MobGII; Sm' Kan'</td>
<td>This work</td>
</tr>
<tr>
<td>GXC2088</td>
<td>GXN1280 harbouring pLAFR1xrvA; Sm' Kan' Tc'</td>
<td>This work</td>
</tr>
<tr>
<td>GXO3098</td>
<td>13751 harbouring pLAFR1xrvA; Sm' Kan' Tc'</td>
<td>This work</td>
</tr>
<tr>
<td>13751hrcV</td>
<td>As 13751, but hrcV:: Tn5; Sm' Kan'</td>
<td>Feng et al. (2001)</td>
</tr>
<tr>
<td>13751xrvAGUS</td>
<td>13751 harbouring pGUSxrvA; Sm' Tc'</td>
<td>This work</td>
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<tr>
<td><strong>Xcc</strong></td>
<td></td>
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<tr>
<td>8523</td>
<td>As wild-type strain 8004, but rpfF:: Tn5lac; Rif' Kan'</td>
<td>Tang et al. (1991)</td>
</tr>
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<td><strong>Plasmids</strong></td>
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<tr>
<td>pGEM-3Zf(+)</td>
<td>Has multiple cloning site (MCS); Amp'</td>
<td>Promega</td>
</tr>
<tr>
<td>pK2K2073</td>
<td>Helper plasmid; Tra+ Mob+ ColE1; Spc'</td>
<td>Leong et al. (1982)</td>
</tr>
<tr>
<td>pLAFR1</td>
<td>Broad-host-range cloning vector; as pLAFR3 but HindIII–EcoRI MCS replaced by that of pUC19; Tc'</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>pLAFR1</td>
<td>Broad-host-range cloning vector; Tc'</td>
<td>Huhny et al. (1989)</td>
</tr>
<tr>
<td>pGXN3400</td>
<td>pLAFR1 containing a 35 kb fragment from the chromosomal DNA of Xoo strain 13751; Tc'</td>
<td>This work</td>
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<tr>
<td>pK18MobGII</td>
<td>Mob+ ColE1 gusA; Kan'</td>
<td>Katzen et al. (1999)</td>
</tr>
<tr>
<td>pK18MobGIIxrvA</td>
<td>pK18MobGII containing a 252 bp internal fragment of xrvA gene; Kan'</td>
<td>This work</td>
</tr>
<tr>
<td>pLAFR1xrvA</td>
<td>pLAFR1 containing a 901 bp fragment including the xrvA gene; Tc'</td>
<td>This work</td>
</tr>
<tr>
<td>pGUSxrvA</td>
<td>pLAFR6 containing an xrvA promoter-gusA fusion fragment</td>
<td>This work</td>
</tr>
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</table>

*Sm', Kan', Tc', Spc' and Rif' indicate resistance to streptomycin, kanamycin, tetracycline, spectinomycin and rifampicin, respectively.

Northern hybridization. Xoo strains were incubated in OB medium to an OD₆₀₀ of 1.0 before being harvested for RNA extraction. For the test in minimal medium XOM2, Xoo strains were first incubated in OB to OD₆₀₀ 1.0 and then the collected cells were washed twice with XOM2, resuspended in XOM2 to OD₆₀₀ 0.1, and cultured in XOM2 to OD₆₀₀ 0.5 before being harvested for RNA extraction. The total RNAs were extracted from the Xoo strains with TRIzol Reagent (Invitrogen). Partial sequences of candidate gene probes were amplified by PCR using the total DNA of the wild-type strain 13751 as the template and the respective primer pairs (Table S1), and then cloned into pGEM-Tc easy vector (Promega) and confirmed by sequencing. The cloned DNA was recovered and used as the probe after restriction digestion and agarose gel electrophoresis. Probes were labelled with [α-³²P]dCTP according to the protocol of the Prime-a-Gene Labelling System (Promega). Unincorporated nucleotides were removed by size-exclusion chromatography using Sephadex G-100 columns. Northern hybridization was carried out in a Hybaid Shake 'n' Stack Hybridization Oven (Thermo) according to the procedures described by Alwine et al. (1977).

Virulence assay and determination of bacterial load in planta and media. Virulence was tested on the hybrid rice cultivar Teyou 63 grown in a greenhouse with a 12 h day–night cycle of illumination at temperatures of 28 °C during the day and 25 °C at night. Bacteria were grown in OB medium at 28 °C with shaking at 200 r.p.m. for 20 h with the specific purpose of reaching the exponential phase of growth. The concentrations of bacterial inocula were adjusted to an OD₆₀₀ of 0.001 (10⁹ c.f.u. ml⁻¹) or 0.1 (10⁸ c.f.u. ml⁻¹). After maintenance at 100 % humidity for 24 h, the inoculated plants were maintained in the growth conditions described above. Lesion length was measured 14 days after inoculation. Thirty leaves were inoculated for each Xoo strain in each treatment by the leaf-clipping method.
(Kauffman et al., 1973). Each treatment was carried out in triplicate per experiment. The same experiment was repeated three times.

The growth of bacteria in rice leaf tissues was measured by homogenizing a group of leaves (five leaves for each sampling) in 9 ml sterile water. Diluted homogenates were plated on OA plates supplemented with streptomycin (for the wild-type), streptomycin plus kanamycin (for the mutant), streptomycin plus kanamycin and tetracycline (for the complemented strain) and streptomycin plus tetracycline (for the overexpression strain GXO3098). The number of bacterial colonies on these plates was counted after incubation at 28 °C for 3 days.

**HR test.** HR was tested in the nonhost castor-oil plant (*Ricinus communis*). The plants were inoculated by infiltrating approximately 20 μl of bacterial suspension (5 × 10⁹ c.f.u. ml⁻¹ or 5 × 10⁶ c.f.u. ml⁻¹) in 10 mM sodium phosphate buffer (5.8 mM Na₂HPO₄ and 4.2 mM NaH₂PO₄, pH 7.0) into the leaves by using a blunt-end plastic syringe. The inoculated plants were maintained in a greenhouse with a 12 h day–night cycle of illumination with a fluorescent lamp and a constant temperature of 28 °C. The HR symptoms were observed and photographed at 10, 20, 30 and 40 h after inoculation. At least ten leaves were inoculated for each treatment per experiment. The same experiment was repeated four times.

**Test of extracellular enzyme activity and EPS production of Xoo strains.** To measure the extracellular activity of endoglucanase, xylanase and amylose, 100 μl culture supernatant was added to 400 μl buffer containing 1% (w/v) carboxymethylcellulose (for endoglucanase), 1% (w/v) oat spelt xylan (for xylanase) or 1% (w/v) soluble starch (for amylose). Reactions were carried out for 30 min at 28 °C. The released reducing sugars were measured as D-glucose equivalent as described by Miller (1959). One unit (U) of the endoglucanase/xylanase/amylose activity was defined as the amount of enzyme releasing 1 μmole of reducing sugar per minute. The activity of protease was quantitatively assayed as described by Swift et al. (1999). To measure EPS production, strains were cultured in 100 ml OB medium containing 2% (w/v) glucose in place of sucrose at 28 °C in a conical flask with shaking at 200 r.p.m. for 4 days. EPS was precipitated from the culture with ethanol, dried, and weighed as described by Tang et al. (1991).

**Measurement of glycogen accumulation by Xoo strains.** Intracellular glycogen accumulation was preliminarily determined by the iodine vapour staining method as described by Chao et al. (2008). Intracellular glycogen accumulation was quantified as follows. Cells of Xoo strains were collected by centrifugation, resuspended in fresh OB medium and lysed by ultrasonication. Trichloroacetic acid was added to the solution to a final concentration of 10% and the mixture was shaken. After centrifugation, the glycogen was precipitated from the collected supernatant by adding an equal volume of 95% ethanol and dried.

**DSF extraction and activity bioassay.** DSF was extracted from ethyl acetate from culture supernatants of Xoo strains grown in OB medium (Barber et al., 1997). The ethyl acetate extracts were evaporated to dryness and samples were resuspended in methanol. The Xcc rpfl mutant strain 8523 (Table 1), which cannot make DSF and is deficient in endoglucanase production, was inoculated into NYGB medium with DSF extracts and grown overnight. The restored extracellular endoglucanase activity produced by strain 8523 was quantitatively measured as described for the measurement of endoglucanase activity above.

**GUS activity assay.** The Xoo strains were cultured in OB and XOM2 media. β-Glucuronidase (GUS) activities were assayed with p-nitrophenyl β-D-glucuronide as the substrate, measuring the A₄₁₅ after the reaction, as described by Jefferson et al. (1986). One unit of GUS activity was defined as the amount of enzyme releasing 1 μmole of p-nitrophenol from p-nitrophenyl β-D-glucuronide per minute. Histochemical GUS staining was performed using 5-bromo-4-chloro-3-indolyglucuronide (X-Gluc) (Promega) as substrate, essentially as described by Jefferson et al. (1987).

**RESULTS**

**Discovery and analysis of the xrvA gene of Xoo**

The discovery of xrvA as a regulator of virulence factor synthesis came from work aimed at identification of genes involved in EPS production of Xoo. The approach was to screen a genomic library of Xoo strain 13751 constructed in the broad-host-range vector pLAFR1 (Huynh et al., 1989; Table 1) for clones that could alter the EPS production of the bacterium. The recombinant plasmids from the genomic library were transferred into the Xoo wild-type strain 13751 by triparental conjugation with selections for Sm<sup>+</sup> (13751) and Tc<sup>+</sup> (pLAFR1 derivatives). One of the 480 transconjugants obtained, which harboured the recombinant plasmid designated pGXN3400, displayed colonies that had less EPS than the wild-type on OB medium agar plates (data not shown). The transconjugant strain also showed significant reduction in virulence in rice (data not shown). These findings suggested that the recombinant plasmid pGXN3400 harbours a gene(s) whose increase in copy number could negatively influence the virulence and EPS production of Xoo. The gene(s) responsible for these effects was located on a 1.3 kb DNA region within the insert DNA of pGXN3400 as determined by subcloning and Tn5 mutagenesis (data not shown). Nucleotide sequence and primer extension analyses of the 1.3 kb DNA region revealed the presence of a transcriptionally active gene that we named xrvA (*Xanthomonas* regulator of virulence) (GenBank accession no. X97866) (Fig. 1).

The xrvA ORF (designated XOO2744, XOO_2588 and PXO_00422 for Xoo strains KACC1033, MAF311018 and PXO99A, respectively) is 402 bp in length and is predicted to encode a protein with 133 amino acids. The XrvA protein is highly homologous with XAC2416 of *Xanthomonas axonopodis* pv. *citri* strain 306 (97% identity), XCV2614 of *Xanthomonas campestris* pv. *vesicatoria* strain 85-10 (97% identity), XorYP_010100010090 of *Xanthomonas oryzae* pv. *oryzicola* strain (96% identity), and XCC2309, XC_1806 and XCCB100_1869 of Xcc strains ATCC33913, 8004 and B100 (91% identity). Protein domain analyses using SMART and the bioinformatic work of Bertin et al. (2001) showed that XrvA possesses an H-NS domain at its C terminus, spanning the 78th to 121st amino acids.

**XrvA is required for the virulence and HR elicitation of Xoo**

To facilitate the functional studies of XrvA, a nonpolar xrvA mutant, named GXN1280 (Table 1), was constructed by homologous suicide plasmid integration (see Methods.
for details). A complemented strain named GXC2088 was also constructed by introducing the recombinant plasmid pLAFRJxrvA, which carries the wild-type xrvA gene (Table 1), into the mutant GXN1280 (see Methods for details). Our previous aCGH (array-based comparative genome hybridization) analyses showed that the pLAFR6 derivative has only one copy in Xcc cells (He et al., 2007). Since plasmid pLAFRJxrvA and pLAFR6 derivatives contain the same replication origin, and Xcc and Xoo are closely related, it is most likely that the complemented strain GXC2088 harbours a single copy of plasmid pLAFRJxrvA and thus possesses only one functional copy of the xrvA gene. Plasmid pLAFRJxrvA was also introduced into the wild-type strain 13751, resulting in strain GXO3098 (Table 1), which harbours an extra xrvA copy carried by the plasmid in addition to the original chromosomal one. All of these strains grew similarly to the wild-type in rich medium OB and minimal medium XOM2 (data not shown).

The virulence of these Xoo strains was tested on the hybrid rice cultivar Teyou 63 by the leaf-clipping inoculation method (Kauffman et al., 1973). Starting inoculum levels were either $10^6$ c.f.u. ml$^{-1}$ (an OD$_{600}$ of 0.001, data not shown) or $10^8$ c.f.u. ml$^{-1}$ (an OD$_{600}$ of 0.1, Fig. 2). Although the mutant strain GXN1280 could still cause obvious bacterial leaf blight symptoms, the symptoms were significantly less severe than those caused by the wild-type (Fig. 2a). At 14 days after inoculation and at both initial inoculum densities, the mean lesion lengths caused by the
mutant were reduced by approximately 37% compared to the wild-type ($P \leq 0.01$ by $t$ test) (Fig. 2b). The mean lesion lengths caused by the complemented strain GXG2088 were however not significantly different from those caused by the wild-type (Fig. 2b). The mean lesion lengths caused by the overexpression strain GXO3098 were also reduced by approximately 35% compared to the wild-type ($P \leq 0.01$ by $t$ test) (Fig. 2b). The wild-type harbouring the vector pLAFRJ showed similar symptoms to the wild-type (data not shown). These findings demonstrated that xrvA is required for the full virulence of Xoo, but suggested that increasing the copy number of xrvA reduced the virulence of the pathogen, which was consistent with the outcome of the initial experiments.

Differences in lesion lengths caused by the different Xoo strains may be associated with differential pathogen spread in the vascular system. To investigate this, we compared the distribution of cells of the mutant strain GXN1280 and the complemented strain GXG2088 in the infected leaves. The suicide plasmid pK18MobGIII (Katzen et al., 1999) used to create the xrvA mutant strain GXN1280 carries a gusA gene. Both strains GXN1280 and GXG2088 express β-glucuronidase (GUS), allowing the bacteria to be visualized within the plant tissue. Histochemical staining of inoculated rice leaves for GUS activity showed that the mutant GXN1280 spread slowly compared with the complemented strain GXG2088 in the vascular system of the infected rice leaves (Fig. 2c). Parallel experiments were carried out with the wild-type reporter strain 13751xrvAGUS, which carries the xrvA promoter-gusA reporter plasmid pGUSXrvA (Table 1). This strain had a similar spread to that of the complemented strain (Fig. 2c). These findings revealed a correlation between the bacterial spread in rice leaves and the symptoms produced by Xoo. However, the total bacterial counts for the wild-type, xrvA mutant, complemented mutant, and overexpression strain GXO3098 within the infected leaves were not significantly different during the 14 days post-inoculation ($P \geq 0.05$ by $t$ test) (data not shown).

To determine whether xrvA has any effect on the elicitation of an HR, we tested the HR-inducing capability of the xrvA mutant in the non-host castor-oil plant. The hrcV mutant of Xoo, strain 13751hrcV (Table 1), was used as a negative control, as this strain is defective in the type III secretion system apparatus and cannot elicit an HR. The results showed that the xrvA mutant strain GXN1280 and overexpression strain GXO3098 elicited an HR that was delayed compared to that caused by the wild-type and the complemented strain GXG2088 (Fig. 3). As expected, the hrcV mutant elicited no visible HR. These observations suggest that xrvA is not absolutely essential for HR induction by Xoo, but contributes to the rapidity of the response.

**XrvA regulates EPS production, DSF synthesis and glycogen accumulation**

The influence of mutation of xrvA on virulence raises the issue of whether XrvA acts to regulate the synthesis of any of the known virulence factors of Xoo. To answer this
question, we examined the effect of xrvA mutation on the production of EPS, extracellular enzymes and DSF as outlined in Methods. The xrvA mutant strain GXN1280 and overexpression strain GXO3098 produced about twofold less EPS compared to the wild-type, while the complemented strain GXC2088 produced a wild-type amount of EPS (Table 2). In parallel, bacterial colonies growing on OB agar plates were assessed for the production of the intracellular polysaccharide glycogen by iodine vapour staining. The wild-type strain 13751 and the complemented strain GXC2088 gave a similar yellowish colour in this assay (data not shown). However, the xrvA mutant strain GXN1280 and overexpression strain GXO3098 stained dark brown, indicating that these two strains contained excess glycogen (data not shown). The amount of intracellular glycogen was then measured quantitatively. As shown in Table 2, the xrvA mutant accumulated almost twofold more glycogen than the wild-type. The level of glycogen in strain GXO3098 was also significantly higher than that in the wild-type (P=0.01 by t test), whereas the complemented strain GXC2088 produced the wild-type level of glycogen (Table 2). These data indicate that xrvA acts to positively regulate EPS production but negatively regulate intracellular glycogen accumulation in Xoo.

The relative levels of the DSF signal molecule in culture supernatants of the various Xoo strains were bioassayed by measuring the restoration of endoglucanase activity to the rpfF mutant strain 8523 of Xcc. This strain cannot make DSF and is deficient in endoglucanase production; addition of exogenous DSF restores endoglucanase production (Barber et al., 1997; Slater et al., 2000). Addition of the DSF preparations from the wild-type and the complemented strain led to a similar significant increase in the endoglucanase production by the mutant 8523, while the preparation from the xrvA mutant strain gave a lesser effect (Fig. 4). Addition of the DSF preparation from strain GXO3098 also led to an increase in endoglucanase production, but the effect was significantly weaker (P=0.05 by t test) than that seen with the wild-type (Fig. 4). These findings demonstrate that xrvA has an influence on DSF production in Xoo.

Although XrvA regulates EPS and DSF production and glycogen accumulation, further experiments revealed that xrvA mutation has no effect on the production of the extracellular enzymes amylase, endoglucanase, protease and xylanase, or on biofilm formation, cell motility, or cell adhesion to inert surfaces (data not shown).

**XrvA regulates the expression of a number of virulence genes in Xoo**

To investigate whether XrvA plays any role in regulating the expression of other known virulence genes such as

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**Table 2. Quantification of extracellular polysaccharide and intracellular glycogen produced by Xoo strains**

Data are the means±SD from triplicate measurements for a representative experiment. The same letters indicate no significant differences; different letters mean significantly different (P=0.01 by t test). The experiment was repeated three times, and similar results were obtained.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Extracellular polysaccharide (g L⁻¹)</th>
<th>Intracellular glycogen (g L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>13751 (wild-type)</td>
<td>17.3±0.013</td>
<td>3.9±0.016</td>
</tr>
<tr>
<td>GXN1280</td>
<td>9.8±0.031</td>
<td>7.6±0.014</td>
</tr>
<tr>
<td>GXC2088</td>
<td>17.1±0.006</td>
<td>4.2±0.030</td>
</tr>
<tr>
<td>GXO3098</td>
<td>11.1±0.041</td>
<td>6.8±0.041</td>
</tr>
</tbody>
</table>
gumB, hrpG, hrpX, rpfB, rpfC, rpfF and rpfG, we employed Northern hybridization analysis to determine the transcript levels of these genes in the xrvA mutant. As outlined above, hrpG, which encodes the master regulator of the hrp regulon in xanthomonads, is expressed in minimal media but is repressed in rich media (Wengelnik et al., 1996). It has been shown that for Xoo, the expression of the hrp genes including the regulatory gene hrpX is induced in XOM2 medium (Tsuge et al., 2002). Therefore, the strains were grown in XOM2 medium for analysis of the transcripts of hrpG and hrpX, and in OB medium for analyses of the other genes. The results showed that the transcript levels of gumB, hrpG, hrpX, rpfC, rpfF and rpfG were lower in the xrvA mutant GXN1280 than in the wild-type (Fig. 5a–f), although the transcript level of rpfB was unaltered (Fig. 5g). The transcript levels of all the tested genes in the complemented strain GXC2088 were almost the same as in the wild-type (Fig. 5). In strain GXO3098, the transcript levels of gumB, hrpX, rpfC, rpfF and rpfG were lower than in the wild-type (Fig. 5a, c, d, e, f). These results suggest that xrvA plays roles in regulating the expression of the virulence genes gumB, hrpG, hrpX, rpfC, rpfF and rpfG in Xoo.

Fig. 4. XrvA regulates DSF biosynthesis of Xoo. DSF extracted from culture supernatants of different strains of Xoo was bioassayed by measuring the restoration of endoglucanase activity to the rpfF mutant strain 8523 of Xcc. Three replicates were done for each experiment and each experiment was repeated three times. Endoglucanase activity is expressed as units per OD600 unit per ml. Means ± so from one experiment are plotted. Similar results were obtained in all experiments.

Fig. 5. Northern hybridization analyses of the transcript levels of virulence-associated genes in Xoo strains 13751 (wild-type), GXN1280 (xrvA mutant), GXC2088 (complemented xrvA mutant) and GXO3098 (wild-type with an additional copy of xrvA) grown in OB (rich) or XOM2 (minimal) medium. The ethidium bromide gel pictures show the loading controls for the RNA samples. Northern analysis was performed for gumB (a), hrpG (b), hrpX (c), rpfC (d), rpfF (e), rpfG (f) and rpfB (g).
The expression of xrvA is enhanced in minimal medium and expressed by bacteria inside the host

The above results demonstrated that xrvA regulates the expression of a number of virulence genes including the key hrp regulatory genes hrpG and hrpX. As described above, the hrp genes of Xanthomonas are induced in minimal media and repressed in rich media. We hypothesized that the expression of xrvA may also be induced in a minimal medium. To verify this, we compared the transcript levels of xrvA in the wild-type strain 13751 grown in the nutrient-rich medium OB and in the hrp-inducing minimal medium XOM2 by Northern hybridization. The result showed that the transcript level of xrvA was significantly elevated under hrp-inducing conditions, i.e. when the bacteria were grown in XOM2 medium (Fig. 6a).

These experiments were extended by examining the expression of xrvA in the wild-type using the xrvA promoter-gusA reporter plasmid pGUSxrvA (Table 1). The result showed that expression of xrvA was nearly 20 times higher in the hrp-inducing XOM2 medium than in the rich medium OB (Fig. 6b, c), which is consistent with the result from the Northern hybridization analyses (Fig. 6a). Moreover, histochemical GUS staining disclosed that xrvA was also expressed when the pathogen was present inside the rice leaves (Fig. 2c).

DISCUSSION

This work adds to the inventory of genes that contribute to the full virulence of Xoo. We have presented genetic evidence to demonstrate that the xrvA gene of Xoo plays an important role in full virulence and normal HR elicitation. Disruption of xrvA led to a significant reduction in virulence, a delay in HR elicitation, a decrease in EPS and DSF production and an increase in glycogen accumulation. Recently, screening of a transposon mutant library of a Korean Xoo strain, KACC10331, in rice also showed that Tn5 insertion in the xrvA gene (XOO2744) led to reduced virulence; however, the mutant was not characterized in further detail (Wang et al., 2008). The deduced protein encoded by xrvA possesses an H-NS domain. These findings are consistent with a body of work that has established a role for hns in the virulence of several bacteria such as E. coli, Er. amylovora, Er. chrysanthemi, Sal. typhimurium and A. pleuropneumoniae (Müller et al., 2006; Hildebrand et al., 2006; Nasser et al., 2001; Harrison et al., 1994; Dalai et al., 2009). The effect of mutation of hns on the ability of Er. amylovora and Er. chrysanthemi to elicit an HR on non-host plants was not assessed, however (Hildebrand et al., 2006; Nasser et al., 2001). Mutation of xrvA in Xoo reduced virulence by about 37% compared to the wild-type. BLASTP analyses showed that there is an XrvA homologue encoded by the ORF XOO3363 in Xoo strain KACC10331 (48% identity), XOO_3168 in Xoo strain MAFF311018 (48% identity), and PXO_01852 in Xoo strain PXO99A (46% identity). Whether there is a functional redundancy between XrvA and its homologue in Xoo needs to be further investigated.

The xrvA mutant GXN1280 and the xrvA overexpression strain GXXO3098 showed a significant reduction in lesion length compared to the wild-type strain but the bacterial populations of these mutants in rice leaves were not significantly different from that of the wild-type. A similar phenomenon was found for a Xoo rpfC mutant, which showed reduced virulence in rice but similar growth as the wild-type strain in planta (Tang et al., 1996). These findings may reflect a more marked contribution of virulence factors to bacterial spread in the tissue rather than to the overall bacterial load.

Although the Xoo xrvA mutant produces less EPS than the wild-type, the mutant accumulates more intracellular...
glycogen. Diverse regulatory effects of hns on polysaccharide synthesis have been reported for bacteria from the Enterobacteriaceae. For example, hns mutants of E. coli are mucoid due to increased production of capsular polysaccharide (Sledjeski & Gottesman, 1995). This increased capsule synthesis results from the increased expression of the gene rcsA, which encodes a positive regulator of the cps genes and is negatively regulated by H-NS (Sledjeski & Gottesman, 1995). Er. amylovora synthesizes two major extracellular polysaccharide species: the complex heteropolymer amylxoran and the fructose-containing homopolymer levan. Er. amylovora carries two copies of hns; one is located on the chromosome and the other on the plasmid pEA29. Mutations in either hns gene led to increased levan production in the presence of sucrose. Interestingly, a difference was observed in the function of the two hns genes in regulation of amylxoran synthesis. Mutation of the chromosomal hns gene caused an increase of EPS production, whereas loss of the plasmid-borne hns gene had no effect (Hildebrand et al., 2006). Nevertheless, overexpression of the hns gene from the Er. amylovora plasmid resulted in decreased production of both amylxoran and levan compared to wild-type (Hildebrand et al., 2006). In Er. chrysanthemi, the production of extracellular polysaccharides, which are required for the efficient bacterial colonization of plants, was also negatively controlled by hns (Nasser et al., 2001).

As described above, HrpG and HrpX are two key hrp regulators in Xanthomonas. HrpG, which is predicted to be a member of the OmpR response regulator family of two-component signal transduction systems, regulates the expression of hrpX, encoding an AraC-type transcriptional activator, which then activates the expression of other hrp operons. Recently, it has been demonstrated that the trh and phoP genes in Xoo positively regulate expression of hrpG, although it is not known whether the regulation is direct or indirect (Tsuge et al., 2006; Lee et al., 2008b). The trh gene encodes a putative transcriptional regulator (Tsuge et al., 2006) and phoP encodes a putative response regulator of two-component regulatory systems (Lee et al., 2008b). Whether trh and phoP influence the expression of other XrvA targets such as gum and rpf has not been reported, however. It will be of interest to further study the functional relationship (if any) between xrvA, trh and phoP in regulating hrpG expression in order to better understand hrp regulatory mechanisms.

The virulence of Xanthomonas also depends upon cell-to-cell signalling mediated by DSF. The RpfC/RpfG two-component system couples the DSF sensing to intracellular regulatory networks through a second messenger, cyclic di-GMP, and a global regulator, Clp. Protein–protein interaction between the DSF synthase RpfF and the sensor RpfC may act as a post-translational mechanism to modulate the biosynthesis of DSF (reviewed by Dow, 2008; He & Zhang, 2008). Mutation of xrvA significantly reduced the DSF production and decreased the transcript levels of rpfF, rpfC and rpfG. Further studies are required to understand whether XrvA regulates these genes directly or indirectly. Nevertheless, to our knowledge, this is the first report of a gene that is involved in regulation of the expression of rpfC, rpfF and rpfG in Xanthomonas spp. It has been established that DSF signalling regulates many different cellular processes in xanthomonads, including the production of the extracellular enzymes endoglucanase, endomannanase and protease, production of EPS and biofilm formation (Dow, 2008). However, our work showed that mutation of xrvA did not significantly influence either the production of extracellular enzymes or biofilm formation by Xoo in vitro (data not shown). This may reflect the fact that inactivation of xrvA reduced but did not completely abolish the synthesis of DSF and expression of the rpf genes (Figs 4 and 5). Whether xrvA has a more pronounced influence on DSF biosynthesis and hence virulence when bacteria are in planta is not known.

Interestingly, in this work, the xrvA mutant strain GXN1280 and the xrvA overexpression strain GXO3098 showed similar phenotypes in virulence, HR elicitation, EPS production and DSF biosynthesis. A similar phenomenon for virulence has been observed in Sal. typhimurium, in which a mutation in the hns gene led to reduced virulence, and introduction of the E. coli hns gene into the wild-type of the pathogen also resulted in attenuation of virulence (Harrison et al., 1994). The reduced expression of hrpG, hrpX, rpfC, rpfF and gumB in the xrvA mutant strain GXN1280, and the decreased expression of hrpX, rpfC, rpfF, rpfG and gumB in the xrvA overexpression strain GXO3098 may partially explain the weakened virulence, delayed HR, and reduced EPS and DSF production of GXN1280 and G XO3098. However, the expression level of hrpX, rpfC, rpfF, rpfG and gumB appeared to be different in GXN1280 and GXO3098, and the expression of hrpG in GXO3098 was not significantly different from that in the wild-type. The molecular basis for these alterations in patterns of gene expression in strains either lacking or overexpressing XrvA is unknown. It is possible that there is an optimal cellular level of XrvA required for expression of virulence genes, and higher levels are inhibitory. Alternatively XrvA may differentially affect the expression of subordinate regulators with opposite regulatory influences on the expression of the virulence-related genes. Much work needs to be done to understand the regulatory mechanisms by which XrvA controls these genes in Xoo. Furthermore, the occurrence of homologues of XrvA in other Xanthomonas spp. warrants study of the role of this H-NS domain protein in the virulence of these pathogens to their diverse host plants.

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