Identification of seven Xanthomonas oryzae pv. oryzicola genes potentially involved in pathogenesis in rice

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Xanthomonas oryzae pv. oryzicola (Xoc) causes bacterial leaf streak (BLS) in rice, an emerging and destructive disease worldwide. Identification of key virulence factors is a prerequisite for understanding the pathogenesis of Xoc. In this study, a Tn5-tagged mutant library of Xoc strain RS105 was screened on rice, and 27 Tn5 mutants were identified that were either non-pathogenic or showed reduced virulence in rice. Fourteen of the non-pathogenic mutants were also unable to elicit the hypersensitive response (HR) in tobacco and were designated Pth⁻/HR⁻ mutants; 13 mutants showed attenuated virulence and were able to induce an HR (Vir⁻/HR⁺). Sequence analysis of the Tn5-tagged genes indicated that the 14 Pth⁻/HR⁻ mutants included mutations in hrcC, hrcT, hrcV, hpaP, hrcQ, hspF, hspG and hspX. The 13 Vir⁻/HR⁺ mutants included tal-C₁₀c-like (a transcriptional activator-like TAL effector), rplC (regulator of pathogenicity factors), oxyR (oxidative stress transcriptional regulator), dsbC (disulfide isomerase), opgH (glucan biosynthesis glucosyltransferase H), rtbA (glucose-1-phosphate thymidlyltransferase), amtR (aminotransferase), purF (amidophosphoribosyltransferase), thrC (threonine synthase), trpA (tryptophan synthase alpha subunit) and three genes encoding hypothetical proteins (Xoryp_02235, Xoryp_00885 and Xoryp_22910). Collectively, the 27 Tn5 insertions are located in 21 different open reading frames. Bacterial growth and in planta virulence assays demonstrated that opgH, purF, thrC, trpA, Xoryp_02235, Xoryp_00885 and Xoryp_22910 are candidate virulence genes involved in Xoc pathogenesis. Reduced virulence in 13 mutants was restored to wild-type levels when the cognate gene was introduced in trans. Expression profiles demonstrated that the seven candidate virulence genes were significantly induced in planta, although their roles in Xoc pathogenesis remain unclear.

INTRODUCTION

Xanthomonas oryzae pv. oryzicola (Xoc) is the causal agent of bacterial leaf streak (BLS), one of the most destructive diseases in high-yield hybrid rice (Oryza sativa L.) (Zhao et al., 2004; Chen et al., 2005; Zou et al., 2006; Wang et al., 2007). The pathogen enters rice leaves through stomata or wound sites and colonizes intercellular spaces in the mesophyll, resulting in water-soaked interveinal lesions that develop into translucent streaks (Chen et al., 2005; Niño-Liu et al., 2006; Zou et al., 2006). Unfortunately, efficient, environmentally friendly strategies to control BLS are lacking.

BLS pathogenesis is totally different from the vascular disease bacterial blight (BB) caused by X. oryzae pv. oryzicola (Xoo). Xoo invades rice plants through water pores or wound sites on leaves, then colonizes the vascular tissues and propagates in the xylem, generating tannish-grey to white lesions along the leaf veins, which leads to yield loss (Ou, 1985). The differences in tissue specificity and symptom development between BLS and BB are presumably due to unique pathogenicity genes that differ in these two bacterial pathogens.

In the last decade, the rapid development of functional and comparative genomics has facilitated the identification of...
pathogenicity-related genes. The whole genome sequences of Xoo strains KACC10331 (Lee et al., 2005), MAFF311018 (Ochiai et al., 2005) and PXO99a (Salzberg et al., 2008), and Xoc strain BLS256 (http://cmr.jcvi.org/tigr-scripts/CMR/GenomePage.cgi?org=Xoc) have revealed differences between Xoc and Xoo (Wren, 2000; Wang et al., 2007). One distinguishing feature between the two pathogens is the prevalence of more transcription activator-like (tal) effector genes of the avrB33/prfA family in Xoc than in Xoo (Yang & White, 2004; Chen et al., 2005; Zou et al., 2005; Niño-Liu et al., 2006); this may contribute to a wider host range for Xoc than for Xoo (Makino et al., 2006). Furthermore, some of the type II and type III secretion system (T2SS and T3SS, respectively) effectors differ in the two pathogens. For example, the T2SS effectors XopO, XopAF, XopAJ and XopAK (http://www.xanthomonas.org/t3e.html) and the T2SS effector extracellular protease EcpA (unpublished data) are present in Xoc but not Xoo. Previous studies also compared the role of pathogenicity-related genes between these two pathovars, including the hrp (hypersensitive response and pathogenicity) (Zou et al., 2006), tal (Yang & White, 2004; Chen et al., 2005; Zou et al., 2005), rpf (regulatory pathogenicity factors) (Tang et al., 1996; Zhao et al., 2011), EPS (extracellular polysaccharide) and LPS (lipopolysaccharide) (Wang et al., 2007; Kim et al., 2009) synthesis genes. However, it remains unclear what novel virulence factors are required by Xoc to determine pathogenesis in rice.

Previous studies have utilized suppression-subtractive hybridization, transcriptional profiling and proteomic analyses to compare virulence genes in Xoo and Xanthomonas campestris pv. campestris (Xcc) (Soto-Suárez et al., 2010; Gonçalves et al., 2010; Hsiao et al., 2011; Zhao et al., 2011). To further understand the Xoc–rice pathosystem, we recently constructed a Tn5 mutant library of Xoc (Zou et al., 2011). In this report, we screened the Tn5 mutants in the library and identified 27 genes involved in Xoc pathogenesis, including seven candidate genes that contribute to virulence in rice.

**METHODS**

**Bacterial strains, culture media and growth conditions.** Strains and plasmids used in this study are listed in Table 1. Xoc strains were grown at 28 °C in NB (1 g yeast extract, 3 g beef extract, 5 g polypeptone, 10 g sucrose; all 1 L), NA (NB with 15 g agar 1 L), NY (NB without beef extract and sucrose), MMX minimal medium [5 g glucose, 2 g (NH4)2SO4, 1 g trisodium citrate dihydrate, 4 g K2HPO4, 6 g KH2PO4, 0.2 g MgSO4.7H2O; all 1 L]. Xoc broth cultures were incubated at 28 °C at 200 r.p.m. Errecsherica coli strains were grown in LB (Luria–Bertani) medium (5 g yeast extract, 10 g NaCl, 10 g tryptone, 15 g agar; all 1 L) at 37 °C (Miller, 1972). Antibiotics were added at the following concentrations (µg mL−1) when required: kanamycin (Kan), 25; rifampicin (Rif), 50; ampicillin (Ap), 100; spectinomycin (Sp), 50; and streptomycin (Sm), 50.

**DNA manipulation.** These were performed as described previously (Sambrook et al., 1989). The conjugation between Xoc and E. coli strains containing plasmids was performed as described by Turner (2004). Kits for isolating genomic and plasmid DNA and for purifying DNA from agarose gels were purchased from Invitrogen. Restriction enzymes and DNA ligases were used according to the manufacturer’s instructions (TaKaRa). PCR primers were purchased from Jinsite and are listed in Supplementary Table S1 (available with the online version of this paper).

**Hypersensitive response (HR) and pathogenicity assays.** Assays for HR and pathogenicity were performed as described previously (Zou et al., 2006). Briefly, Xanthomonas cells were grown in NB broth with appropriate antibiotics at 28 °C and 200 r.p.m. for 16 h, when cells approached the exponential phase of growth. Bacterial cells were then harvested by centrifugation, washed twice and resuspended in sterile water to OD600 0.3 (approx. 1 × 109 c.f.u. ml−1). Bacteria were then infiltrated into tobacco leaves (cv. Xanthi) using a needleless syringe or inoculated into leaves of adult rice plants (cv. Shanyou63, susceptible to BLS, 2 months old) using leaf-needling for lesion length measurement or a needleless syringe for evaluating water-soaking symptoms in rice seedlings (2 weeks old). All plants were grown and maintained in a greenhouse as described previously (Wang et al., 2007). Plant phenotypes were scored 24 h post-inoculation (p.i.) for HR in tobacco, 3 days p.i. for water-soaking symptoms in rice seedlings, and 14 days p.i. for lesion lengths in adult rice. Five leaves were inoculated for each independent experiment and each treatment was repeated at least three times.

**Southern blot assay.** Genomic DNA of Xoc strains was extracted as described by Leach et al. (1990). DNA samples were digested with PstI for 4 h and separated in 1.2% agarose gels in TAE buffer for 6 h at 80 V. Subsequently, the digested DNA was transferred to a Hybond-N+ nylon membrane and hybridized with a 500 bp PCR probe amplified from Tn5 and labelled with digoxigenin (PCR DIG probe synthesis kit, Roche). Hybridization was detected using the DIG luminescence detection kit (Roche). The wild-type Xoc strain RS105 was used as a negative control. Southern hybridization experiments were conducted twice to confirm the results.

**Determination of the sequences flanking the transposon by TAIL-PCR.** Genomic DNA sequences flanking the Tn5 insertion in selected mutants were determined by thermal asymmetrical interlaced PCR (TAIL-PCR) (Yuan et al., 2009; Zou et al., 2011). For TAIL-PCR, two sets of nested primers specific to the two ends of the transposon and six arbitrary degenerate primers were used (Supplementary Table S1). TAIL–PCR products were gel-purified, cloned into PMD18-T (TaKaRa) and sequenced. DNA sequences were analysed by evaluating homology to the hpr clusters of Xoc RS105 (GenBank accession nos AY875714 and AY875714), the genome sequence of Xoc strain BLS256 (http://cmr.jcvi.org/tigr-scripts/CMR/GenomePage.cgi?org=Xoc; and GenBank accession no. CP003057, Ye et al., 2006).

**Complementation of the tested mutants.** For complementation analysis, DNA fragments containing the intact genes or operons with native promoters were amplified by PCR using genomic DNA of Xoc RS105 as a template (primer sets listed in Supplementary Table S1). After sequence verification, the amplified DNA fragments were cloned into the pHM1 vector at HindIII. KpnI, SalI or EcoRI sites to create recombinant plasmids (Table 1). Recombinant plasmids were transformed into the corresponding Tn5 insertion mutants by electroporation (Zou et al., 2006), and the transformants harbouring the recombinant plasmids were screened on NA plates with appropriate antibiotics. Selected transformants were verified by PCR amplification using the corresponding primer pairs (Supplementary Table S1).

**Determination of bacterial growth ability.** To determine their ability to multiply in planta, Xanthomonas suspensions (1 × 108 c.f.u. ml−1) were infiltrated into the intercellular spaces of fully expanded leaves of rice (2 weeks old, cv. Shanyou63) with a needleless syringe at...
Table 1. Strains and plasmids used in this study

Ap<sup>r</sup>, ampicillin resistant; Km<sup>r</sup>, kanamycin resistant; Rif<sup>r</sup>, rifampicin resistant; Sp<sup>r</sup>, spectinomycin resistant; Sm<sup>r</sup>, streptomycin resistant.

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Rice suspension cells. *Oryza sativa* subsp. *indica* cv. Shanyou63, which is susceptible to *Xoc* strain RS105, was used for callus induction. Seeds were dehulled and sterilized in 70% ethanol for 10 min, transferred to a solution containing 50% commercial bleach with a few drops of Tween 20 for 30 min, and then soaked in 1% HgCl<sub>2</sub> for 15 min. Sterilized seeds were washed five times with sterile distilled water and incubated on N6 medium (Chu, 1978) containing 2,4-dichlorophenoxyacetic acid (2,4-D) (5 mg l<sup>−1</sup>) at 28 °C in darkness. Actively growing calli were then selected and transferred to liquid N6 medium containing 5 mg 2,4-D l<sup>−1</sup> and 1 mg kinetin (KT) l<sup>−1</sup>. Rice calli were incubated in the dark and transferred by subculturing at 7 day intervals at a dilution of 1:5 (inoculum: fresh medium). Generally, large amounts of rice suspension cells were obtained after 4–5 weeks of subculturing; at this time, single round rice cells could be observed under the microscope.

Three locations per leaf. Three leaf discs (0.8 cm in diameter) were harvested with a cork borer from each infiltrated area. After sterilization in a solution containing 70% ethanol and 30% sodium hypochlorite, the leaf discs were homogenized in 1 ml sterile distilled water. Diluted homogenates were plated on NB agar supplemented with rifampicin (wild-type *Xoc* with rifampicin) or spectinomycin plus kanamycin (for complementing strains) to determine the c.f.u. cm<sup>−2</sup>. Bacterial c.f.u. were counted after incubation at 28 °C for 3 days, and the c.f.u. per cm<sup>2</sup> leaf area was calculated.

To investigate bacterial growth in vitro, NB, NY and MMX media were used. *Xanthomonas* cells were pre-incubated in 5 ml NB medium for 16–20 h at 28 °C, 200 r.p.m. until OD<sub>600</sub> 0.6. Bacterial cells were then collected by centrifugation, washed twice and resuspended to OD<sub>600</sub> 0.05 in 20 ml NB, NY and MMX media. For each time point, 200 μl each culture was removed and the OD<sub>600</sub> was measured. The data presented are representative of three independent experiments.
RESULTS

Identification of Tn5 mutants involved in Xoc pathogenicity and the HR

To identify key virulence genes or factors involved in Xoc pathogenicity, we previously generated a library of Tn5 insertion mutants (Zou et al., 2011), and 27 mutants showed altered pathogenicity or virulence compared with the wild-type strain RS105. To confirm the attenuated pathogenicity in rice and HR induction in tobacco, we reinoculated these 27 mutants to rice seedlings (2-week-old seedlings of cv. Shanyou63). The phenotype of lesions and lesion lengths were scored 3 and 14 days p.i. and compared with the wild-type RS105 (Fig. 1a, Table 2). In subsequent experiments, the mutants were individually inoculated to adult rice (2 months old) by leaf-needling and infiltrated into tobacco for a more precise assessment of pathogenicity and ability to induce the HR, respectively. Fourteen mutants were non-pathogenic in rice and defective in the ability to trigger an HR in tobacco (designated Pth−/HR−) (Table 2). The remaining 13 mutants showed reduced virulence in rice, but retained the ability to elicit an HR in tobacco (Vir−/HR+) (Fig. 1, Table 2). With the exception of Mxoc0729, the Vir− mutants generated significantly smaller lesion lengths than the wild-type (P = 0.01, t test) (Fig. 1b). Thus, the results indicated that the 27 mutants could be divided into two groups: Pth−/HR− and Vir−/HR+ (Table 2).

Sequence analysis of disrupted genes

To further characterize the 27 Tn5 insertion mutants, we first evaluated whether each mutant contained a single Tn5 insertion. Genomic DNA of the mutants and wild-type was digested with PstI and hybridized with the 500 bp probe derived from Tn5. Southern blot analysis indicated that each mutant contained a single Tn5 insertion (Fig. 2a).

It has been confirmed that the ability of Xoc to trigger an HR in non-host tobacco and to be pathogenic in rice is determined by the hrp clusters that encode components of the T3SS (Zou et al., 2006; Li et al., 2011a, b). To determine whether or not the 14 Pth−/HR− mutants contain Tn5 insertions in the hrp gene cluster, we employed long PCR to amplify the 5 kb hrp-hrc-hpa regions of Xoc RS105 (Supplementary Table S1) and the 14 Pth−/HR− mutants. After sequencing the 14 amplified fragments (data not shown), we demonstrated that Tn5 insertions mapped to hrcC (mutants Mxoc0397 and Mxoc1472), hrcF (Mxoc0529), hrcV (Mxoc1114, Mxoc1042, Mxoc1021 and Mxoc1481), lpaP (Mxoc0536), hrcQ (Mxoc0139 and Mxoc0665), hpf (Mxoc0548), hrgP (Mxoc1546) and hrgX (Mxoc0707 and Mxoc1314) (Table 2, Fig. 3a). Sequence analysis also indicated that the transposon had inserted at different locations within single hrp genes (e.g. see hrpV; Fig. 3a).

To locate the Tn5 insertion in the 13 Vir−/HR+ Tn5 mutants (Fig. 1, Table 2), we employed a TAIL-PCR technique, as described previously (Yuan et al., 2009; Zou et al., 2011), and amplified regions flanking the transposon in these mutants. After sequencing the flanking fragments, the transposon insertions were mapped and oriented by aligning the sequences with the completed genome of Xoc BLS256 strain as the reference. The results are presented in Table 2 and Fig. 3(b–n). The genes disrupted by Tn5 could be divided into two groups. Group I includes six genes with known functions in other phytopathogens (Fig. 3, Table 2), including the tal-C10c-like gene, which encodes a TAL effector (http://www.xanthomonas.org/t3e.html) in the mutant Mxoc0729; rpfC (Xoryp_10980), a regulatory pathogenicity factor identified in Xcc (He & Zhang, 2008) in Mxoc1232; oxyR (Xoryp_04700), an oxidative stress transcriptional regulator characterized in the phytopathogen Xylella fastidiosa (Toledo et al., 2011) in Mxoc1304; dsbC (Xoryp_18770) encoding a disulfide isomerase involved in Xcc virulence (Jiang et al., 2008) in Mxoc0525; rfbA (Xoryp_18935), a glucose-1-phosphate thymidlyltransferase required for mycobacterial growth (Qu et al., 2007) in Mxoc0028; and amtR (Xoryp_18935), an aminotransferase associated with virulence in Pseudomonas syringae (Park et al., 2010), in Mxoc0091. It is important to note that oxyR, rfbA and amtR have not been previously reported in the virulence of Xanthomonas species.

Group II contains seven genes not previously reported in the virulence of phytopathogenic bacteria but are highly conserved in Xanthomonas species (Fig. 3, Table 2). This group includes opgH (Xoryp_03285), which encodes glucan biosynthesis glucosyltransferase H in Mxoc0852; purF (Xoryp_05420) an amidophosphoribosyltransferase in Mxoc0601; thrC (Xoryp_10705) a threonine synthase in Mxoc0104; trpA (Xoryp_14510) a tryptophan synthase alpha subunit in Mxoc0305, and the other three genes,
Xoryp_02235, Xoryp_00885 and Xoryp_22910, which encode hypothetical proteins in Mxoc0236, Mxoc0065 and Mxoc0761, respectively.

Since the genome sequence of Xoc RS105 is unavailable, we used the genome of Xoc BLS256 as a reference. To determine whether RS105 and BLS256 vary in the sequence of the genes identified in this study, 12 primer pairs (Supplementary Table S1; the tal-C10c-like gene was not included) were designed and used to amplify the Tn5-disrupted genes in the mutants; the wild-type RS105 were used as the controls. In (b), values are the means ± SD from three repeats, each with five leaves; hatched bars, mutant strains; black bars, complemented strains. **P = 0.01; *P = 0.05; paired, two-tailed Student t test for each mutant relative to the wild-type. 1, Mxoc1304; 2, Mxoc0236; 3, Mxoc0028; 4, Mxoc0525; 5, Mxoc0104; 6, Mxoc0091; 7, Mxoc0350; 8, Mxoc0065; 9, Mxoc1232; 10, Mxoc0729; 11, Mxoc0761; 12, Mxoc0852; 13, Mxoc0601; 14, RΔhrcV; 15, RS105.

Fig. 1. Pathogenicity tests in rice (cv. Shanyou63, 2 weeks old) by infiltration of candidate virulence-reduced mutants of Xoc at OD<sub>600</sub> 0.3 (approx. 1 x 10<sup>9</sup> c.f.u. ml<sup>-1</sup>) with needleless syringes (a) and by inoculation of the same candidates at the same concentration in rice (cv. Shanyou63, 2 months old) by using the leaf-needling method (b). Water-soaked symptoms were photographed 3 days p.i. in rice seedlings. The lesion length in adult rice was measured 14 days p.i. The T3SS-deficient mutant RΔhrcV and the wild-type RS105 were used as the controls. In (b), values are the means ± SD from three repeats, each with five leaves; hatched bars, mutant strains; black bars, complemented strains. **P = 0.01; *P = 0.05; paired, two-tailed Student t test for each mutant relative to the wild-type. 1, Mxoc1304; 2, Mxoc0236; 3, Mxoc0028; 4, Mxoc0525; 5, Mxoc0104; 6, Mxoc0091; 7, Mxoc0350; 8, Mxoc0065; 9, Mxoc1232; 10, Mxoc0729; 11, Mxoc0761; 12, Mxoc0852; 13, Mxoc0601; 14, RΔhrcV; 15, RS105.

Complementation analysis of 13 Xoc Tn5 mutants

It has been demonstrated that transposon insertions can cause polar effects on downstream genes (Wang et al., 2007). To exclude the possibility that the reduced virulence observed for group II mutants resulted from polar effects
**Table 2. Tn5-inserted mutants of *X. oryzae pv. oryzicola* strain RS105**

Pth", No pathogenicity in rice; HR", no HR induction in tobacco; HR", HR induced in tobacco. Xoo, *X. oryzae pv. oryzae*; Xcc, *X. campestris pv. campestris*; Xac, *X. axonopodis pv. citri*; Xcv, *X. campestris pv. vesicatoria*.

<table>
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<tr>
<th>Mutants</th>
<th>Rice lesion length (cm)*</th>
<th>HR induction</th>
<th>ORFs in BLS256†</th>
<th>Inserted position‡</th>
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Table 2, cont.

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*Significant (P<0.01, t test) reduction in virulence by different mutants is shown by different letters.

The genome sequence of Xoc BLS256 strain (http://cmr.jcvi.org/tigr-scripts/CMR/GenomePage.cgi?org=Xoc) is used as the reference.

dPosition is given relative to the first nucleotide of the open reading frame.

*| Significant (P<0.01, t test) reduction in virulence by different mutants is shown by different letters.

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impaired with respect to growth in MMX compared with the wild-type (Fig. 5b). Mutants Mxoc0104 (thrC), Mxoc0350 (trpA) and Mxoc0601 (purF) did not grow in MMX (Fig. 5b). Growth of the complemented strains CMxoc0104, CMxoc0350 and CMxoc0601 was identical to the wild-type RS105 in MMX (data not shown), indicating that the growth could be restored by thrC, trpA and purF, respectively, in trans.

Expression of seven candidate virulence genes is induced in planta

Real-time quantitative RT-PCR was used to assess transcriptional activity of these seven genes in the wild-type RS105 grown in four conditions: nutrient-rich medium (NB), minimal medium (MMX), rice suspension cells and rice leaves (see Methods). Expression of the seven candidate virulence genes was significantly higher (2.1- to 21.4-fold higher; \(P=0.01\), t test) when the pathogen interacted with rice suspension cells or rice leaves than when grown in NB and MMX, respectively (Fig. 6). Interestingly, transcription of the seven genes was significantly higher in rice suspension cells than in rice leaves (Fig. 6). The results suggest that the seven genes (opgH, trpA, thrC, purF, Xoryp_02235, Xoryp_00885 and Xoryp_2291) are potentially involved in virulence, and their expression is dependent on the interaction of Xoc with rice.

Fig. 2. Southern blot analysis (a) and PCR verification (b) of Tn5 insertions in candidate virulence-reduced mutants of Xoc. Genomic DNA of the mutants was digested with PstI, and probed with a 500 bp fragment amplified by PCR from the Tn5 transposon. To confirm that Tn5 is inserted in the target of the mutant, the corresponding primer pairs (Supplementary Table S1), based on the genome sequence of Xoc BLS256, for the entire genes and their promoter regions were explored to amplify the corresponding DNA fragments from the wild-type RS105 and the Tn5-tagged mutants. The PCR-amplified products in the tested mutants should be 1221 bp larger than those in the wild-type. For detection of the Tn5-inserted tal-C10c-like (Xoryp_21665) in Mxoc0729, one primer from Xoryp_21665 and another from the Tn5 transposon (Supplementary Table S1) were used. Mxoc0729 gave rise to a corresponding PCR product, but the wild-type did not. Marker, λ-EcoT14 DNA ladder. 1, Mxoc1304; 2, Mxoc0236; 3, Mxoc0028; 4, Mxoc0525; 5, Mxoc0104; 6, Mxoc0091; 7, Mxoc0350; 8, Mxoc0065; 9, Mxoc1232; 10, Mxoc0729; 11, Mxoc0761; 12, Mxoc0852; 13, Mxoc0601; R, RS105; 15, EcoT14; 16, DL2000.
DISCUSSION

In the present study, a Tn5-tagged mutant library of Xoc was screened, and 21 genes were identified with roles in pathogenicity or virulence. In addition to genes with known functions [e.g. tal (tal-C10c-like), rpfC, amtR, oxyR, dsbC and rfbA], seven candidate virulence genes were identified, including oggH, purF, thrC, trpA, Xoryp_02235, Xoryp_00885 and Xoryp_22910; the latter three genes encode hypothetical proteins highly conserved in Xanthomonas species (Table 2).

Oxidative stress plays a crucial role in plant defence against the invasion and colonization of pathogens (Baker & Orlandi, 1995). oxyR, the gene disrupted in mutant Mxoc1304, encodes a transcriptional regulator of oxidative stress (Table 2). OxyR, a LysR-type transcriptional regulator, is a DNA-binding protein that governs bacterial transcriptional regulation. The presence of oxyR and other oxidative stress-related genes in the mutant library suggests that oxidative stress is a significant factor in the pathogenicity of Xoc. The identification of candidate virulence genes and the role of oxidative stress in plant defence provide valuable insights into the mechanisms of pathogenesis and virulence in Xoc.
oxidative stress and scavenges reactive oxygen species (ROS) (Lee et al., 2004; Toledo et al., 2011; Zheng et al., 1998; Maddocks & Oyston, 2008). OxyR has been studied in E. coli (Christman et al., 1989), Xylella fastidiosa (Toledo et al., 2011), and Xanthomonas campestris pv. phaseoli (Kullik et al., 1995). The highly conserved N-terminal

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Fig. 4. Bacterial populations of the seven novel gene mutants, the corresponding complementary stains and the wild-type strain RS105 in rice (cv. Shanyou63). Bacteria were recovered from the inoculated leaves at 1 day intervals for 4 days p.i. by infiltration of the above strains at OD600 0.3 (approx. $1 \times 10^8$ c.f.u. ml$^{-1}$) with needleless syringes, and were homogenized in sterile water. The homogenates were diluted and plated on NA plates with appropriate antibiotics. Bacterial c.f.u. were counted after incubation at 28 °C for 3 days. Data are the mean±SD from three repeats, and each experiment was repeated three times. Mutant, ■; complemented mutant, ○; wild-type RS105, ▲. Tn5 insertions were at Mxoc0852 (a), Mxoc0601 (b), Mxoc0104 (c), Mxoc0350 (d), Mxoc0236 (e), Mxoc0065 (f) and Mxoc0761 (g).

Fig. 5. Growth curves of Xoc strains in NY (no sugar) medium (a) and MMX minimal medium (b). The wild-type strain RS105 was used as a control. Strains were inoculated into 20 ml liquid medium and incubated at 28 °C with shaking at 200 r.p.m. Aliquots (200 μl each) were taken in triplicate at intervals, and bacterial growth was determined by measuring OD 600 against the medium blank. Data presented are means±SD from a representative experiment; similar results were obtained in two other independent experiments. Mxoc0104, •; Mxoc0350, ■ (dotted line); Mxoc0601, ▲; Mxoc0852, ○; Mxoc0065, △; Mxoc0236, □; Mxoc0761, x; RS105, ■ (solid line).
DNA-binding domain and C-terminal regulatory domain in OxyR may enable plant-pathogenic bacteria to sense intracellular ROS and activate the transcription of genes involved in scavenging oxidative species. The disruption of oxyR in Xoc (mutant Mxoc1304) impaired virulence in rice (Fig. 1), which suggests that successful infection and colonization of rice requires Xoc to respond appropriately to oxidative stress.

Two genes with known functions, rfbA and amtR, which were identified in the Vir− mutants Mxoc0028 and Mxoc0091, encode glucose-1-phosphate thymidyltransferase and an aminotransferase, respectively. RfbA catalysts the conversion of glucose-1-phosphate to dTDP-rhamnose (Lerouge & Vanderleyden, 2002); the latter is a component of the O-antigen of LPS in Gram-negative bacteria including Xanthomonas. In Mycobacterium tuberculosis, rfbA was identified as a virulence factor that is essential for mycobacterial growth and cell morphology (Qu et al., 2007). Lipopolysaccharides are well-documented virulence determinants in mammalian pathogens (Lerouge & Vanderleyden, 2002) and also function in the Xcc infection process (Newman et al., 2002). However, a function for rfbA in LPS production by Xoc has not yet been demonstrated. Regarding the aminotransferase encoded by amtR, the final product catalysed by the enzyme flows into the tricarboxylic acid (TCA) cycle (Mailloux et al., 2007). Bacterial pathogens utilize intermediates of the TCA cycle as carbon sources when there is insufficient hexose (Magne et al., 1997; Oh et al., 2002; Tang et al., 2005). The reduced virulence and growth observed for the Mxoc0091 mutant (Figs 1 and 4) may be due to an impaired ability to acquire nutrients in rice.

In Gram-negative bacteria, two independent metabolic pathways are involved in disulfide bond formation in extracytoplasmic proteins. One is the DsbA/DsbB oxidation pathway and the other is the isomerization/reduction pathway (Messens & Collet, 2006; Łasica & Jaguszyn-Krynicka, 2007). DsbA is essential for multiple processes in mammalian pathogens including toxin production, adhesion, motility, extracellular enzyme production and the T3SS (Urban et al., 2001; Stenson & Weiss, 2002; Ha et al., 2003; Tinsley et al., 2004; Hiniker & Bardwell, 2004). dsbB mutants of Xcc exhibited reductions in virulence, the HR, cell motility and bacterial growth in planta (Jiang et al., 2008). Furthermore, the dsbB mutation impaired the T2SS, T3SS and flagellar assembly (Jiang et al., 2008). In the present study, the dsbC mutant (Mxoc0525) was significantly reduced with respect to virulence and growth in rice (Figs 1 and 4). dsbC encodes a disulfide isomerase belonging to the isomerization/reduction pathway. Periplasmic DsbC functions as a disulfide bond isomerase to reduce misfolded proteins (Hiniker et al., 2003; Lee et al., 2008). The mutation in the dsbC gene had multiple effects on extracellular enzymes and cell motility in Erwinia carotovora (Vincent-Sealy et al., 1999) and impaired extracellular enzyme formation in Pseudomonas aeruginosa (Urban et al., 2001). It remains unclear whether DsbC could function similarly in Xoc, and this warrants further study.

The most compelling group of genes identified in this study are the seven candidate virulence factors (Table 2). opgH encodes glucan biosynthesis glucosyltransferase H which is associated with cell-surface adhesion of Streptococcus mutans (Cross et al., 2007). OpgH in Xoc is 96% identical to OpgH in Xer (Minsavage et al., 2004), which is highly homologous to the previously reported pathogenicity-related HrpM protein of P. syringae pv. syringae (Mukhopadhyay et al., 1988; Penaloz-Vazquez et al., 2010) and OpgH of E. chrysanthemi (Cogez et al., 2001). Purf is generally considered to be a key enzyme in the purine de novo biosynthetic pathway (Shimaoka et al., 2007). The mutation in purF is considered to render the organism auxotrophic as determined by an inability to grow on MMX minimal medium (Qian et al., 2005). It may not function directly to reduce virulence in planta, but instead may impact survival due to the subsequent auxotrophy. thrC, which encodes a threonine synthase, is required for the production of the toxic intermediate homoserine in Cryptococcus neoformans and Saccharomyces cerevisiae. In these two organisms, thrC mutants suppressed serum sensitivity, which is a consequence of homoserine accumulation. Serum survival is critical for dissemination, an important virulence determinant in S. cerevisiae (Kingsbury & McCusker, 2010). The protein product of trpA, which encodes a tryptophan synthase alpha subunit, converts indole-3-glycerol phosphate into tryptophan, an essential amino acid for Salmonella typhimurium (Kriechbaumer et al., 2003).
et al., 2008). Thus, the trpA mutation in Xoc may attenuate virulence by limiting nutrient availability for the pathogen. However, the actual functions of OpgH, PurF, ThrC and TrpA in Xoc pathogenicity warrant further investigation.

The three remaining genes encode hypothetical proteins. The translational product of Xoryp_02235 has a recognizable AcCoA-syn-alpha domain typical of acetyltransferase (GNAT) family proteins in X. campestris pv. vasculorum (NCPPB702) (data not shown) and is 96–99% identical to the homologue in other xanthomonads (Table 2). Xoryp_022910 encodes a conserved hypothetical protein without any identified conserved domains; similarly, it is 80–96% identical to homologues in other Xanthomonas species. Xoryp_00885 also encodes a hypothetical protein of unknown function that is highly conserved in Xanthomonas species (Table 2). Interestingly, Xoryp_02235 and Xoryp_00885 are highly induced (~20-fold) in planta compared with expression in NB and MMX minimal medium (Fig. 5). The transcriptional activation of these genes suggests a potential role in the Xoc–rice interaction, a theory that will be investigated in future studies.

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Seven candidate genes for Xoc virulence


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