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The ColRS system of *Xanthomonas oryzae* pv. *oryzae* is required for virulence and growth in iron-limiting conditions

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SUMMARY

Xanthomonas oryzae pv. *oryzae*, the causal agent of bacterial blight of rice, produces siderophores only under iron-limiting conditions. We screened 15 400 mTn*5*-induced mutants of *X. oryzae* pv. *oryzae* and isolated 27 mutants that produced siderophores even under iron-replete conditions. We found that the mTn*5* insertions in 25 of these mutants were in or close to six genes. Mutants with insertions in five of these genes [*colS*, XOO1806 (a conserved hypothetical protein), *acnB*, *prpR* and *prpB*] exhibited a deficiency for growth on iron-limiting medium and a decrease in virulence. Insertions in a sixth gene, XOO0007 (a conserved hypothetical protein), were found to affect the ability to grow on iron-limiting medium, but did not affect the virulence. Targeted gene disruptants for *colR* (encoding the predicted cognate regulatory protein for ColS) also exhibited a deficiency for growth on iron-limiting medium and a decrease in virulence. *colR* and *colS* mutants were defective in the elicitation of hypersensitive response symptoms on the nonhost tomato. In addition, *colR* and *colS* mutants induced a rice basal defence response, suggesting that they are compromised in the suppression of host innate immunity. Quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis demonstrated that a functional ColRS system is required for the optimal expression of several genes encoding components of the type 3 secretion system (T3SS) of *X. oryzae* pv. *oryzae*. Our results demonstrate the role of several novel genes, including *colR/colS*, in the promotion of growth on iron-limiting medium and the virulence of *X. oryzae* pv. *oryzae*.

INTRODUCTION

Xanthomonas oryzae pv. *oryzae*, the causal agent of bacterial blight of rice, uses a diverse array of virulence functions: surface polysaccharides; adhesins; a type 2 secretion system and its secreted proteins, which include enzymes involved in the degradation of rice cell walls; a type 3 secretion system (T3SS) and its secreted effectors, which function inside rice cells to suppress host innate immunity and up-regulate the expression of host susceptibility factors; a diffusible signalling factor (DSF)-mediated cell–cell signalling system; iron uptake functions; a phytase-like protein, etc. (Buttner and Bonas, 2010; Nino-Liu *et al*., 2006).

Several regulatory functions are also required for the virulence of *X. oryzae* pv. *oryzae*. One of these regulatory functions is HrpG, a transcriptional activator of the OmpR family of two-component systems, which controls the expression of the genes for T3SS and its secreted proteins. The HrpG protein exerts its effects by up-regulating the expression of another transcriptional activator, called HrpX (Buttner and Bonas, 2010). The PhoPQ system is required for virulence and controls the expression of the *hrpG* (hypersensitive response and pathogenicity G) gene (Lee *et al*., 2008). In addition to the PhoPQ system, the H-NS protein XrvA and Trh (a member of the GntR regulator family) also regulate the expression of *hrpG* (Feng *et al*., 2009; Tsuge *et al*., 2006). The PhoPQ system and another two-component system, RaxRH, are required for Ax21 (a peptide that induces rice XA21-mediated immunity) activity (Lee *et al*., 2008, 2009). The *rpfF* (regulation of pathogenicity factor F) gene-mediated DSF system has been shown to promote the virulence of *X. oryzae* pv. *oryzae* by facilitating iron uptake (Chatterjee and Sonti, 2002).

Xanthomonas oryzae pv. *oryzae* encodes an *xss* (*Xanthomonas* siderophore synthesis) operon, which is required for the biosynthesis of a vibrioferrin-type siderophore, and a *feoABC* operon-encoded Feo system for ferrous iron uptake (Pandey and Sonti, 2010). Siderophore-deficient mutants and *feoB* mutants grow poorly in iron-limiting conditions, but only the *feoB* mutant is virulence deficient (Pandey and Sonti, 2010). *Xanthomonas oryzae* pv. *oryzae* produces siderophores only in iron-limiting conditions. A mutation in the *fur* (ferric uptake regulator) gene causes the constitutive production of siderophores (Subramoni and Sonti, 2005). In order to identify additional regulatory functions that influence iron uptake in *X. oryzae* pv. *oryzae*, we screened 15 400 mTn*5*-induced mutants for the ability to produce siderophores even on iron-replete medium. Such mutants, which we refer to as siderophore overproducers, or *sop*, mutants, may possess mutations in either regulatory functions or certain iron uptake processes whose suboptimal functioning results in siderophore overproduction as a compensatory measure. Using thermal

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Table 1 Summary of sequence analyses of open reading frames (ORFs) disrupted in *Xanthomonas oryzae* pv. *oryzae* siderophore overproducer (*sop*) mutants.

ORF (amino acids)*	No. of mTn*5* insertions in/close to gene; phenotypes of mutants†	Predicted function	Presence of conserved domain‡
XOO0007 (397)	15; Vir⁺, Lir⁻	Unknown	Putative Zn-dependent protease, contains TPR (tetratrico tetrapeptide repeats), 5.2e-03§
XOO0891 (591)	1; Vir⁻, Lir⁻	PrpR; propionate catabolism regulatory protein	PrpR_N superfamily; propionate catabolism activator, 4.24e-45§
XOO0892 (298)	1; Vir⁻, Lir⁻	PrpB; 2-methyl isocitrate lyase	PEP phosphonomutase and related enzymes, 4e-85§
XOO1207 (450)	4; Vir⁻, Lir⁻	ColS; two-component system sensor protein	Histidine kinase A (dimerization/phosphoacceptor domain), e-13§ Histidine kinase-like ATPases, 2.2e-10§
XOO1806 (289)	2; Vir⁻, Lir⁻	Unknown	Orn/DAP/Arg decarboxylases family 2 signature 2; partial, 0.0¶
XOO2862 (863)	2; Vir⁻, Lir⁻	AcnB; aconitate hydratase	AcnB, aconitase B catalytic domain, 0e+00§ Aconitase B swivel domain, 5.88e-54§

*Size of the ORF in amino acids.

†Vir⁺ and Vir⁻ indicate proficiency and deficiency for virulence, respectively. Lir⁻ indicates deficiency for growth on low-iron medium.

‡The *E*-value obtained from the conserved domain analysis.

§Using the National Center for Biotechnology Information (NCBI) Conserved Domain Search program.

¶Using the EBI InterProScan program.

asymmetric interlaced polymerase chain reaction (TAIL PCR) and, subsequently, PCR with gene-specific primers, we were able to localize the insertions in 25/27 *sop* mutants to be in/close to six genes. One of the affected genes is *colS*, which is the sensor component of the two-component system, ColRS. Targeted mutagenesis of the *colR* and *colS* genes, accompanied by complementation analysis, demonstrates that this two-component system is required for virulence on rice, an ability to grow on iron-limiting medium and optimal functioning of T3SS.

RESULTS

Isolation of *X. oryzae* pv. *oryzae sop* mutants

An *X. oryzae* pv. *oryzae* mTn*5* library was generated (see Experimental procedures) using *in vivo* transposon mutagenesis of strain BXO43 (our laboratory wild-type strain). A total of 15 400 mTn*5* mutants were screened on peptone–sucrose agar-chrome azurol sulphonate (PSA-CAS) plates. After repeated screening, 27 *sop* mutants that exhibited consistent phenotypes of siderophore overproduction (Fig. 1A), without exhibiting apparent growth defects, were selected for further characterization.

Identification of mutated genes

Using mTn*5*-specific as well as arbitrary primers (Table S1, see Supporting Information), TAIL PCR was performed to amplify the flanking regions of mTn*5* insertions, and the PCR products thus obtained were sequenced. The expected locations of the transposon insertions in the genome were obtained by performing BLAST searches with the *X. oryzae* pv. *oryzae* KACC10331 genome sequence (Lee *et al*., 2005). Subsequently, locus-specific primers were designed to confirm that the insertions were indeed at the expected locations. The locations of insertions in 25 mutants were confirmed by a combination of locus- and transposon-specific primers, and these were found to be in or close to different open reading frames (ORFs). For these 25 mutants, the complete amino acid sequences of *X. oryzae* pv. *oryzae* ORFs that carried the mTn*5* insertion in/close to the genes were analysed to determine homology with previously characterized proteins in sequence databases and to detect conserved domains (Table 1).

Fifteen of the 25 *sop* mutants carried insertions either in a gene annotated as XOO0007, which encodes for a conserved hypothetical protein, or in the intergenic region between this gene and the adjacent *tonB* gene (XOO0008). Other genes mutated in *sop* mutants included: *colS* (XOO1207), which encodes a putative two-component sensor protein; *acnB* (XOO2862), which encodes a putative aconitase (Acn); *prpB* (XOO0892), which encodes a putative methyl isocitrate lyase involved in propionate catabolism; *prpR* (XOO0891), which encodes a putative regulator of *prp* genes; and XOO1806, which encodes a conserved hypothetical protein. Southern analysis was performed on 10 *sop* mutants and all were found to have single-copy insertions of mTn*5* (data not shown). These 10 mutants included the following: two insertions each in the *colS*, *acnB*, XOO0007 and XOO1806 genes, and one insertion each in the *prpB* and *prpR* genes.

Siderophore overproduction by *sop* mutants is suppressed by iron supplementation

In order to determine whether the siderophore overproduction phenotype is caused by a defect in sensing the levels of intracellular iron, the secretion of siderophores was monitored on PSA-CAS plates supplemented with 30 μM $FeSO_4$. All of the *sop* mutants stopped secreting siderophores under these conditions.

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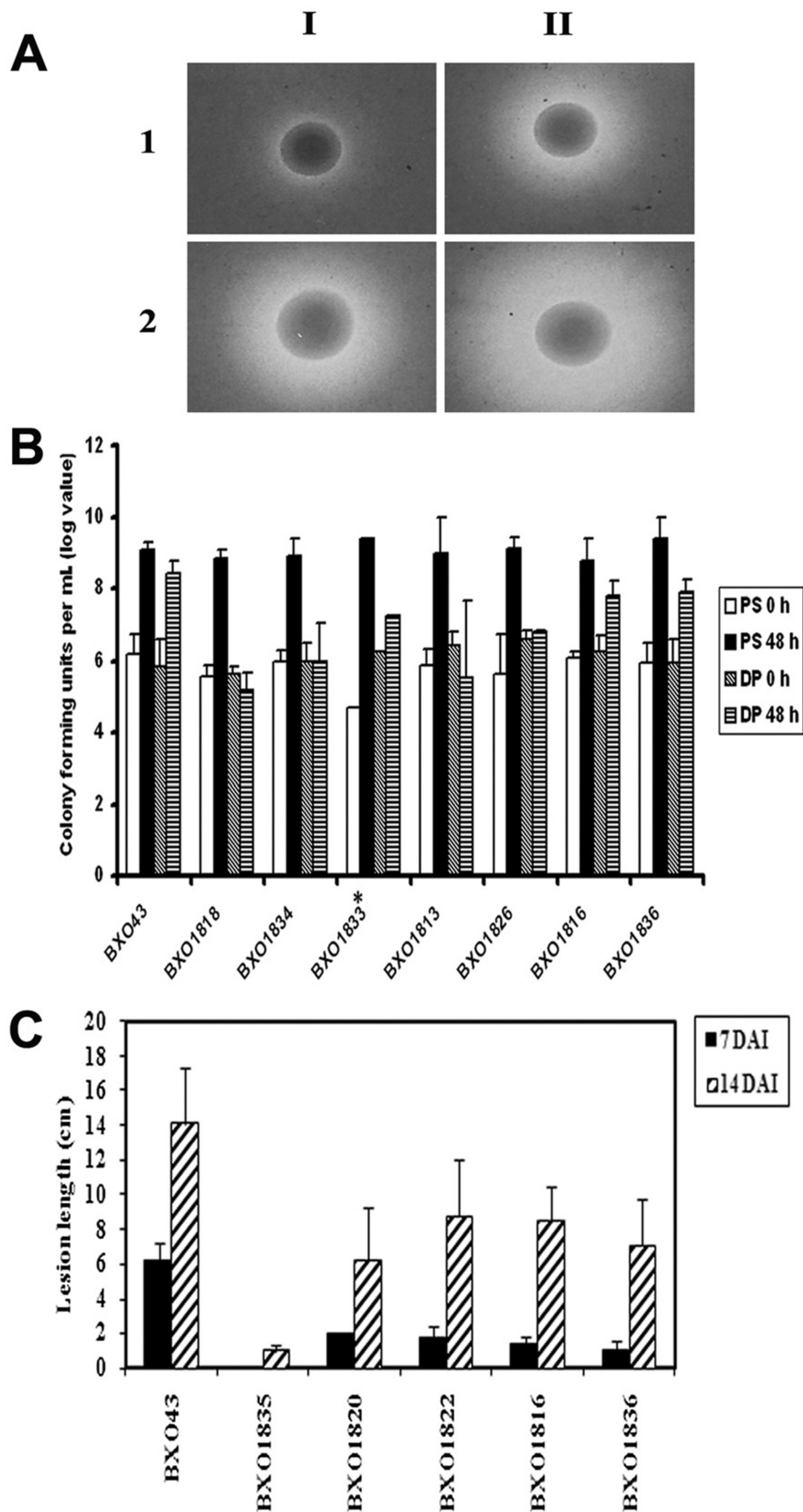
A
I
II
1
2
B
Colony forming units per mL (log value)
12
10
8
6
4
2
0
PS 0 h
PS 48 h
DP 0 h
DP 48 h
BXO43
BXO1818
BXO1834
BXO1833*
BXO1813
BXO1826
BXO1816
BXO1836
C
Lesion length (cm)
20
18
16
14
12
10
8
6
4
2
0
7DAI
14DAI
BXO43
BXO1835
BXO1820
BXO1822
BXO1816
BXO1836

Fig. 1 Siderophore phenotype, growth on iron-limiting medium and virulence properties of siderophore overproducer (*sop*) mutants of *Xanthomonas oryzae* pv. *oryzae*. (A) Siderophore production phenotype of *X. oryzae* pv. *oryzae* strains: 1, BXO43 (wild-type strain); 2, BXO1816 (a representative *sop* mutant). The strains were grown on: I, peptone–sucrose agar plates containing chrome azurol sulphonate (PSA-CAS), considered as an iron-replete condition; II, PSA-CAS + 40 μM 2,2′-dipyridyl, an iron chelator (PSA-CAS + DP), considered as an iron-limiting condition. Siderophore production is indicated by the presence of an extended halo around the colony. BXO43 produces siderophore only under iron-limiting conditions, whereas BXO1816 produces siderophore in iron-replete as well as iron-limiting conditions. (B) *Xanthomonas oryzae* pv. *oryzae sop* mutants are deficient for growth on iron-limiting medium. Cell numbers were estimated in *X. oryzae* pv. *oryzae* cultures grown in peptone–sucrose (PS) and PS + 140 μM DP at 0 h and after 48 h of growth. Data are the mean log colony-forming unit (cfu) values ± standard deviation from three independent experiments. Strains are as indicated on the *x* axis: BXO43, BXO1818 (*colS1*::mTn*5*), BXO1834 (*XOO1806-2*::mTn*5*), BXO1833 (*acnB2*::mTn*5*), BXO1813 (*zxx301*::mTn*5*, a mutation in the XOO0007–XOO0008 intergenic region), BXO1826 (*XOO0007-6*::mTn*5*), BXO1816 (*prpR1*::mTn*5*) and BXO1836 (*prpB1*::mTn*5*). Student's two-tailed *t*-test for independent means was performed and the growth of each of the mutants on iron-limiting medium was significantly different ($P < 0.05$) from the wild-type strain. The asterisk indicates that the cell numbers given for this strain are expressed as the mean of the values obtained from two experiments. (C) Virulence phenotypes of *X. oryzae* pv. *oryzae sop* mutants. Inoculations were performed on leaves of 40-day-old glasshouse-grown plants of the susceptible rice cultivar Taichung Native-1 (TN-1). Lesion lengths were measured 7 and 14 days after inoculation (DAI). The mean and standard deviation of 15 replicate measurements are given. The results from one experiment are presented. Similar results were obtained in two other independent experiments. Student's *t*-test showed that the values for the mutants were significantly different ($P < 0.05$) from that of the wild-type strain at both 7 and 14 days. Strains are: BXO43, BXO1835 (*colS4*::mTn*5*), BXO1820 (*XOO1806-1*::mTn*5*), BXO1822 (*acnB1*::mTn*5*), BXO1816 and BXO1836.

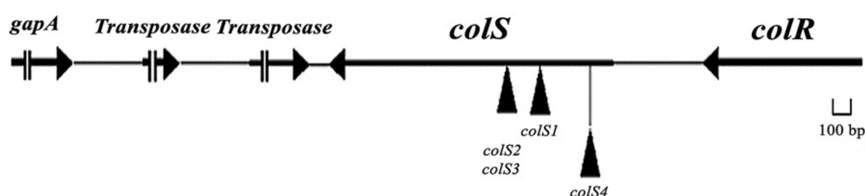

Fig. 2 Location of mTn*5* insertions and gene organization in the *Xanthomonas oryzae* pv. *oryzae* genomic region containing the *colS* gene. The filled triangles represent the locations of mTn*5* insertions. The arrows indicate the transcriptional orientations of the genes. The bold lines indicate open reading frames (ORFs) and the lighter lines indicate intergenic regions. The *colS2* (BXO1823) and *colS3* (BXO1824) mutants carry the mTn*5* insertion at the same site in the *colS* gene. Immediately downstream of *colS* are ORFs coding for putative transposase genes, followed by *gapA* encoding for glyceraldehyde-3-phosphate dehydrogenase. These genes are located in the opposite transcriptional orientation with respect to *colS*. The gene organization given here is as specified by Lee *et al*. (2005) in the genome of *X. oryzae* pv. *oryzae* strain KACC10331.

Evaluation of *X. oryzae* pv. *oryzae sop* mutants for virulence and growth on iron-limiting medium

The insertions in the XOO0007 gene are concentrated in the 3′ region of the ORF and two insertions are in the intergenic region between the XOO0007 and XOO0008 (*tonB*) genes (Fig. S1, see Supporting Information). These mutants are defective for growth on iron-limiting medium (data are shown for two mutants, BXO1813 and BXO1826, in Fig. 1B), but retain wild-type levels of virulence (data not shown). The strains carrying insertions in the XOO1806 gene are defective for growth on iron-limiting medium (data shown only for BXO1834 in Fig. 1B) and exhibit reduced virulence (data shown only for BXO1820 in Fig. 1C).

The *sop* mutants (BXO1822 and BXO1833) with insertions in the *X. oryzae* pv. *oryzae* homologue of *acnB* (XOO2862) exhibit a virulence deficiency (data shown only for BXO1822 in Fig. 1C) and a growth defect on iron-limiting medium (data shown only for BXO1833 in Fig. 1B). The two *sop* mutants (BXO1816 and BXO1836) with insertions in the *prpR* (XOO0891) and *prpB* (XOO0892) genes exhibit a deficiency for virulence (Fig. 1C) and for growth on iron-limiting medium (Fig. 1B).

Four *sop* mutants (BXO1818, BXO1823, BXO1824 and BXO1835) carried insertions in the *colS* gene (Fig. 2). The predicted protein product of *colS* is 450 amino acids in length and exhibits significant similarity to the sensor component of bacterial two-component regulatory systems. In particular, it exhibits 37% identity and 55% similarity (across a 264-amino-acid segment in the C-terminal region) to ColS, the sensor component of the *Pseudomonas fluorescens* WCS365 two-component system that promotes the colonization of the roots of several plants, including potato, tomato, radish and wheat (Dekkers *et al*., 1998). The ColS protein of *X. oryzae* pv. *oryzae* exhibits homology (80% identity and 87% similarity) to the predicted products of the XCC3106, XC_1050 and XAC3249 genes of *Xanthomonas campestris* pv. *campestris* strains ATCC33913 and 8004, and *Xanthomonas citri* ssp. *citri* strain 306, respectively.

All four *colS* mutants (BXO1818, BXO1823, BXO1824 and BXO1835) isolated in this study exhibit virulence deficiency. The data for BXO1835 are shown in Fig. 1C, but similar results were obtained with the other three mutants. These mutants grow as well as the wild-type strain on peptone–sucrose (PS) medium, but show severe growth deficiency on iron-limiting medium (Fig. 1B).

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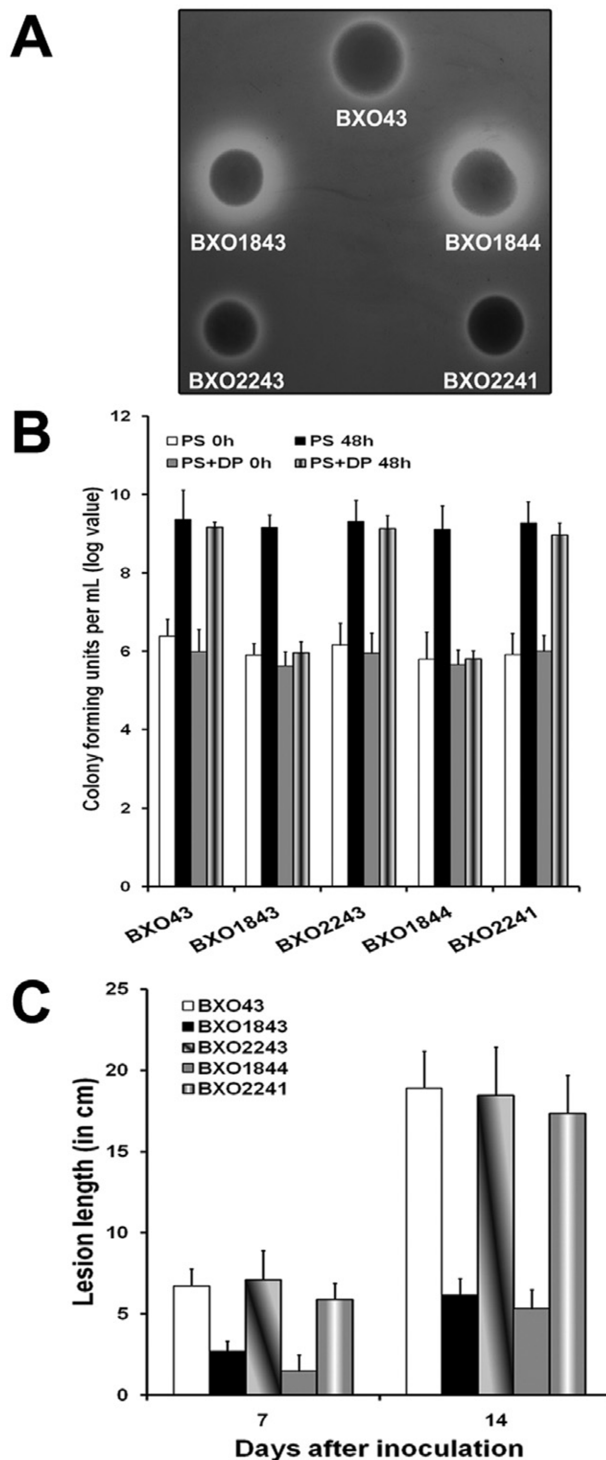

Fig. 3 Siderophore production, virulence and growth phenotype of a *colR* mutant of *Xanthomonas oryzae* pv. *oryzae*. *Xanthomonas oryzae* pv. *oryzae colR* and *colS* mutants were constructed by integration of suicide plasmid pK18*mob*. (A) Siderophore production is assayed as the presence of a halo around colonies growing on peptone–sucrose agar (PSA) medium containing chrome azurol sulphonate (CAS). Strains: BXO43 (wild-type strain), BXO1843 (*colR1*::pK18*mob*), BXO1844 (*colS5*::pK18*mob*), complemented strain BXO2243 (BXO1843/pAP24, ColR$^+$) and complemented strain BXO2241 (BXO1844/pAP25, ColS$^+$). The pAP24 and pAP25 plasmids carry the cloned *X. oryzae* pv. *oryzae colR* and *colS* genes, respectively. A *colR* mutant overproduces siderophores. Wild-type levels of siderophore are restored by the addition of pAP24. (B) A *colR* mutant is deficient for growth on iron-limiting medium. Cell numbers were estimated in *X. oryzae* pv. *oryzae* cultures grown in peptone–sucrose (PS) medium and PS + 140 μM 2,2′-dipyridyl (DP) at 0 h and after 48 h of growth. Data are the mean log cfu values ± standard deviation from three independent experiments. (C) A *colR* mutant is severely virulence deficient. Inoculations were performed on leaves of susceptible rice cultivar Taichung Native-1 (TN-1), as described in Experimental procedures. Lesion lengths were measured 7 and 14 days after inoculation. The mean and standard deviation of 15 replicate measurements are given. The results from one experiment are presented. Similar results were obtained in independent experiments.

Data for the BXO1818 strain are shown (Fig. 1B), but similar results were obtained with the other three mutants.

A *colR* mutant exhibits a Sop phenotype and is deficient for virulence and growth on iron-limiting medium

The *colR* gene (XOO1208) is present upstream of the *colS* gene (Fig. 2), with an intergenic interval of 430 bp in the genome of *X. oryzae* pv. *oryzae* strain KACC10331 (Lee *et al*., 2005). An ISXo1 element is present in this intergenic region in the genome of *X. oryzae* pv. *oryzae* strain PXO99^A (Salzberg *et al*., 2008). The predicted protein product of *colR* is 246 amino acids in length and exhibits significant similarity to the regulator components of bacterial two-component systems. In particular, it exhibits 58% identity and 75% similarity throughout the length of the protein to ColR, the regulatory component of the *P. fluorescens* WCS365 two-component system. The ColR protein of *X. oryzae* pv. *oryzae* also exhibits homology (99% identity) to the products of the XCC3107, XC_1049 and XAC3250 genes of *X. campestris* pv. *campestris* strains ATCC33913 and 8004, and *X. citri* ssp. *citri* strain 306, respectively.

In order to ascertain the phenotypes associated with a *colR* mutation, an *X. oryzae* pv. *oryzae colR* mutant (strain BXO1843) was generated by homologous integration of a recombinant pK18*mob* vector containing an internal fragment of the *colR* gene, as described in Experimental procedures. An additional *X. oryzae* pv. *oryzae colS* mutant (BXO1844) was generated using a similar strategy. The BXO1843 (*colR* mutant) and BXO1844 (*colS* mutant) strains exhibit a Sop phenotype (Fig. 3A). The complementing plasmids carrying the *colR* gene (pAP24) and *colS* gene (pAP25) were introduced into the BXO1843 and BXO1844 strains, respectively, to create the complemented strains, BXO2243 (ColR$^+$) and BXO2241 (ColS$^+$). The BXO2243 and BXO2241 strains do not produce siderophores on PSA-CAS medium (Fig. 3A).

In PS medium, no significant difference in growth yield was observed between the wild-type strain BXO43 and the BXO1843

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(*colR* mutant) and BXO1844 (*colS* mutant) strains. The BXO1843 and BXO1844 strains exhibit a deficiency for growth when cultured in iron-limiting conditions (Fig. 3B). However, the complemented strains, BXO2243 ($ColR^+$) and BXO2241 ($ColS^+$), exhibit a level of cell density similar to that observed for BXO43 under these conditions. These results indicate that the ColRS system of *X. oryzae* pv. *oryzae* is required for growth under iron limitation.

The virulence phenotypes of BXO43 (wild-type strain), BXO1843 (*colR* mutant), BXO2243 ($ColR^+$), BXO1844 (*colS* mutant) and BXO2241 ($ColS^+$) were determined by inoculating rice leaves and assessing lesion lengths at 7 and 14 days after inoculation. At both time points, the BXO1843 and BXO1844 strains showed significantly smaller lesions when compared with those produced by the BXO43, BXO2243 and BXO2241 strains (Fig. 3C). However, we observed that the BXO1843 and BXO1844 strains produced slightly elongated lesions at the 14-day time point. Bacteria were re-isolated from the leading edge of the lesion in rice leaves. These bacteria were found to exhibit the wild-type phenotype with respect to siderophore production and virulence (data not shown). In addition, these strains were susceptible to kanamycin (Km), indicating that they were $ColR^+$ (BXO1845) and $ColS^+$ (BXO1846) revertants that arose after loss of the integrated plasmid as a result of homologous recombination events involving internally duplicated sequences of the *colR* and *colS* genes, respectively. It is relevant to note that reversions were not observed with any of the *colS*::mTn*5* insertion mutants, providing additional evidence that $ColR^+$ and $ColS^+$ revertants observed following inoculation with BXO1843 and BXO1844 are caused by homologous recombination events that result in the loss of integrated plasmid. Taken together, these results indicate that the ColRS system is essential for the virulence of *X. oryzae* pv. *oryzae* on rice.

Effect of *colR* and *colS* mutations on the expression of genes encoding iron uptake functions

Xanthomonas oryzae pv. *oryzae* encodes an *xss* operon that is required for the biosynthesis of siderophores, as well as an *feoABC* operon-encoded Feo system for ferrous iron uptake (Pandey and Sonti, 2010). The effect of *colR* and *colS* mutations on the expression of the *feoB* (encodes the ferrous iron transporter) and *xssE* (encoded in the *xss* operon) genes was assessed using real-time quantitative PCR, as described in Experimental procedures. When compared with the wild-type strain BXO43, expression of the *feoB* gene was reduced significantly ($P < 0.05$) in the BXO1843 (*colR* mutant) and BXO1844 (*colS* mutant) strains. However, expression of the *xssE* gene was enhanced significantly ($P < 0.05$) in the *colR*/*colS* mutants (Table 2). In the complemented strains BXO2243 ($ColR^+$) and BXO2241 ($ColS^+$), the expression level of the *feoB* and *xssE* genes was comparable with that of BXO43 (Table 2). These results indicate that the *X. oryzae* pv. *oryzae* ColRS system is required for optimal expression of the *feoB* gene.

Table 2 Effect of *Xanthomonas oryzae* pv. *oryzae colR* and *colS* mutations on expression of *xssE* and *feoB* genes.

Strain	Fold expression change ± SD*	
	xssE	*feoB*
BXO1843 (*colR* mutant)	3.13 ± 0.37†	0.38 ± 0.20†
BXO2243 (complemented *colR* mutant)	1.04 ± 0.32‡	1.42 ± 0.46‡
BXO1844 (*colS* mutant)	2.19 ± 0.73†	0.47 ± 0.25†
BXO2241 (complemented *colS* mutant)	0.85 ± 0.21‡	1.31 ± 0.49‡

*The fold expression change (mutant/wild type) (complemented mutant/wild type) was calculated using $2^{-\Delta\Delta Ct}$ as described in Experimental procedures. Mean ± standard deviation (SD) of fold expression change for each gene is presented from three biological replicates.

†Indicates that the fold expression changes are significantly different (two-tailed *t*-test, $P < 0.05$) with respect to the wild-type strain.

‡Indicates that the fold expression changes are not significantly different (two-tailed *t*-test, $P < 0.05$) with respect to the wild-type strain.

colR and *colS* mutants are defective in the elicitation of the hypersensitive response (HR) and expression of certain *hrp* genes

The role of the *X. oryzae* pv. *oryzae colR* and *colS* genes in the elicitation of HR symptoms was evaluated by infiltrating bacterial suspensions in leaves of tomato plants, as described in Experimental procedures. Wild-type strain BXO43 elicited HR symptoms in tomato leaves. The BXO1843 (*colR* mutant) and BXO1844 (*colS* mutant) strains exhibited substantially reduced HR. The complemented strains BXO2243 ($ColR^+$) and BXO2241 ($ColS^+$) regained the ability to elicit wild-type levels of HR (Fig. 4). As expected, BXO2012, a strain that is T3SS deficient [mutated for the *hrpB6* (*hrcN*) gene; Jha *et al*. (2007)] did not elicit an HR (Fig. 4).

The HR phenotype associated with the *colR* and *colS* mutants suggests a role for the ColRS system in the expression of *hrp* genes. In *X. oryzae* pv. *oryzae*, the *hrp* gene cluster consists of more than 20 genes representing six operons (*hrpA*–*hrpF*) (Lee *et al*., 2005; Ochiai *et al*., 2005; Oku *et al*., 2004; Zhu *et al*., 2000). In order to determine the role of the ColRS system in the regulation of *hrp* genes, the expression profile of one gene from each of these operons was assessed using real-time quantitative PCR in *colR* and *colS* mutants. These genes were as follows: *hrcC* (*hrpA* operon), *hrcT* (*hrpB* operon), *hrcU* (*hrpC* operon), *hrcR* (*hrpD* operon), *hrpE1* (*hrpE* operon) and *hrpF* (*hrpF* operon). We found that, when compared with BXO43, the expression of *hrcU*, *hrcR*, *hrpE1* and *hrpF* genes was decreased significantly ($P < 0.05$) in the BXO1843 (*colR* mutant) and BXO1844 (*colS* mutant) strains. In the complemented strains BXO2243 ($ColR^+$) and BXO2241 ($ColS^+$), the expression level of the *hrcU*, *hrcR*, *hrpE1* and *hrpF* genes was comparable with that of BXO43 (Fig. 5A,B). These results indicate that the *X. oryzae* pv. *oryzae* ColRS system is required for the optimal expression of the *hrcU*, *hrcR*, *hrpE1* and *hrpF* genes.

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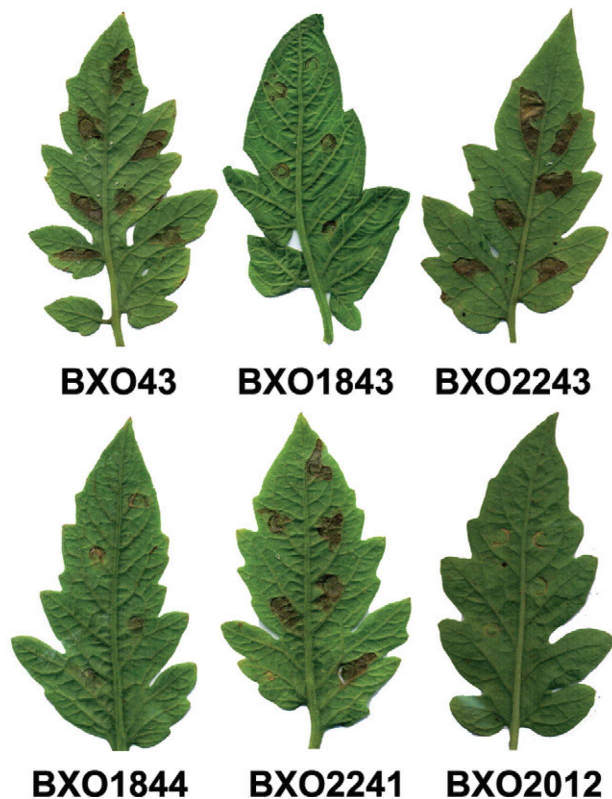

Fig. 4 *colR* and *colS* mutants of *Xanthomonas oryzae* pv. *oryzae* are deficient in the induction of a hypersensitive response (HR) on tomato. The leaves of tomato plants were infiltrated with bacterial suspensions as described in Experimental procedures. HR symptoms were observed 16 h after infiltration. Wild-type strain BXO43 and the complemented strains BXO2243 (ColR+) and BXO2241 (ColS+) induced HR symptoms on tomato leaves, but BXO1843 (*colS* mutant), BX01844 (*colR* mutant) and BXO2012 (*hrpB6*::*bla*; a type 3 secretion system-deficient mutant) did not.

However, disruption of the *colR* and *colS* genes did not affect the expression of the *hrcC* and *hrcT* genes, which are encoded in the *hrpA* and *hrpB* operons (Fig. 5A,B).

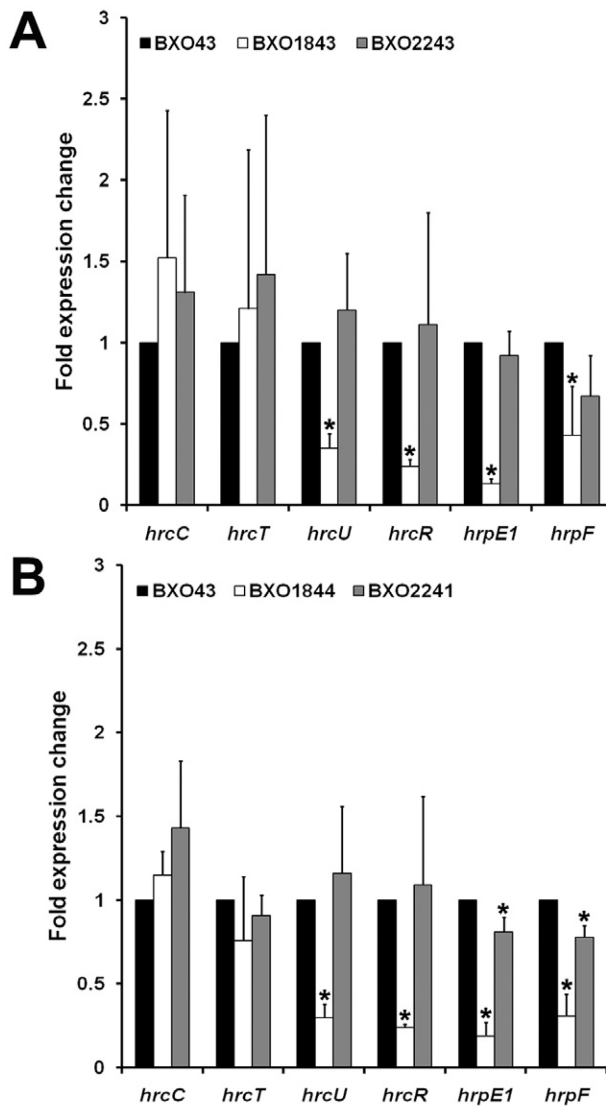

Fig. 5 The *colR* and *colS* mutants of *Xanthomonas oryzae* pv. *oryzae* exhibit reduced expression of *hrcU*, *hrcR*, *hrpE1* and *hrpF* genes. In comparison with the wild-type strain (BXO43), the fold expression change of *hrp* genes was measured in BXO1843 (*colR* mutant) and the complemented strain BXO2243 (ColR+) (A) and BXO1844 (*colS* mutant) and the complemented strain BXO2241 (ColS+) (B). Real-time quantitative polymerase chain reaction analysis was performed for *hrp* genes with cDNA synthesized using total RNA extracted from *hrp*-inducing XOM2 medium-grown cultures. The *hrp* genes analysed are indicated on the *x* axis. The *16S rRNA* gene was used as an endogenous control. The fold expression change (mutant/wild type) (complemented mutant/wild type) was calculated using $2^{-\Delta\Delta Ct}$. The mean ± standard deviation of the fold expression change for each gene is presented from three biological repeats. Asterisks indicate that the fold expression change values are significantly different (two-tailed *t*-test, $P < 0.05$) with respect to the wild-type strain.

Xanthomonas oryzae pv. *oryzae colR* and *colS* mutants are inducers of rice basal defence responses

The above data suggest that the expression of some T3SS genes is down-regulated in *colR* and *colS* mutants. In an earlier study, we have demonstrated that a T3SS-deficient mutant (BXO2012) of *X. oryzae* pv. *oryzae* induces rice basal defence responses, seen as callose deposition (Jha *et al*., 2007). Therefore, we assessed the ability of *colR* and *colS* mutants to induce callose deposition. As expected, minimal callose deposits were observed in rice leaves infiltrated with BXO43 (Fig. 6A-a,6B). When compared with BXO43, the BXO1843 (*colR* mutant) and BXO1844 (*colS* mutant) strains induced significantly ($P < 0.001$) larger amounts of callose deposition (Fig. 6A-b,d,6B). The numbers of

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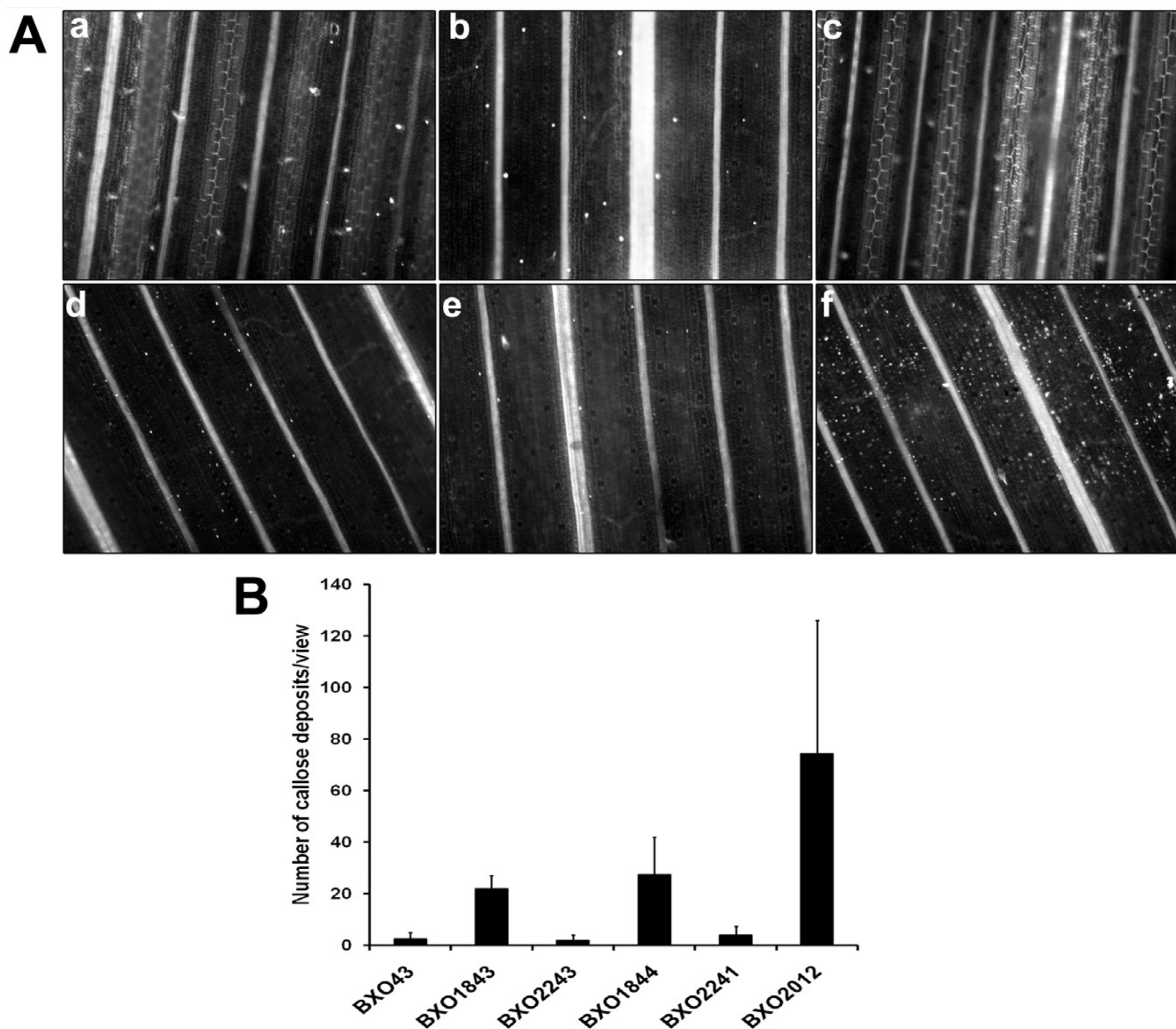

Fig. 6 *colR* and *colS* mutants of *Xanthomonas oryzae* pv. *oryzae* induce callose deposition in rice leaves. (A) Representative images of rice leaves infiltrated with BXO43 (wild-type strain) (a), BXO1843 (*colR* mutant) (b), BXO2243 (ColR$^+$, complemented strain) (c), BXO1844 (*colS* mutant) (d), BXO2241 (ColS$^+$, complemented strain) (e) and BXO2012 (type 3 secretion system-deficient mutant) (f), and examined under a fluorescence microscope after staining with aniline blue. Each image represents a leaf area of approximately 0.56 μm^2. (B) Quantification of callose deposits per field of view (~ 0.56 μm^2) after infiltration of rice leaves with *X. oryzae* pv. *oryzae* strains. The numbers of callose deposits obtained with the BXO1843 and BXO1844 strains were significantly (two-tailed *t*-test, $P < 0.001$) higher than those observed for BXO43. Data were collected from at least three leaves in each experiment, and three different viewing areas from the infiltrated region of each leaf. Similar results were obtained in four independent experiments.

callose deposits in the leaves infiltrated with complemented strains BXO2243 (ColR$^+$) and BXO2241 (ColS$^+$) were comparable with those observed in leaves infiltrated with BXO43 (Fig. 6A-c,e,6B). Taken together, these data indicate that the *colR*/*colS* mutants are inducers of rice basal defence responses. However, the numbers of callose deposits induced by either BXO1843 or BXO1844 are lower than those induced by a T3SS-deficient mutant (BXO2012; Fig. 6A-f,6B).

DISCUSSION

We have carried out a genome-wide screen to identify *X. oryzae* pv. *oryzae* mutants that exhibit siderophore production under iron-replete conditions. We have called them 'siderophore over-producer' (*sop*) mutants. Of the 27 mutants isolated, the mTn*5* insertions in 25 mutants were localized to be in or close to six genes. The siderophore overproduction phenotype of all of the *sop*

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mutants can be reversed by iron supplementation in the medium, indicating that this phenotype is not caused by a defect in sensing the levels of intracellular iron. This is in contrast with the previously identified *X. oryzae* pv. *oryzae fur* regulatory mutant, which fails to shut off siderophore production, even on supplementation with extracellular iron (Subramoni and Sonti, 2005).

Four *sop* mutants carried insertions in the *colS* gene, which codes for a putative *X. oryzae* pv. *oryzae* two-component sensor protein. Targeted mutagenesis of *colR*, the cognate regulator, also results in a Sop phenotype. The *X. oryzae* pv. *oryzae colR* and *colS* mutants are deficient for growth in iron-limiting conditions, but exhibit no growth deficiency in PS medium. This suggests that the ColRS system of *X. oryzae* pv. *oryzae* may be required for the optimal expression of genes involved in some aspects of iron uptake/metabolism. Quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis indicates that the expression of the *feoB* gene is down-regulated in *colR* and *colS* mutants. It is quite possible that the growth deficiency of the *colR* and *colS* mutants on iron-limiting medium is caused by reduced expression of the *feoB* gene. The overexpression of siderophores in *colR* and *colS* mutants, as evident from the phenotype on CAS medium and enhanced expression of the *xssE* gene, is likely to be a compensatory measure for the reduced expression of *feoB*.

The *X. oryzae* pv. *oryzae colR/colS* mutants are defective in the elicitation of an HR on the nonhost plant, tomato. When compared with the wild-type, the *colR*/*colS* mutants elicit enhanced levels of callose deposition, which is an indicator of the induction of rice innate immunity. Both phenotypes are indicative of a deficiency in T3SS of *X. oryzae* pv. *oryzae*, and suggest that there may be reduced expression of *hrp*/*hrc* genes in *colR/colS* mutants. The *hrp* genes of xanthomonads have been reported to be organized into six operons, namely the *hrpC*/*D*/*E*/*F*/*A*/*B* operons. We examined the effect of the mutation of *colR* and *colS* genes of *X. oryzae* pv. *oryzae* on the expression of one gene from each of these operons. Mutational inactivation of either *colR* or *colS* genes leads to reduced expression of *hrcU*, *hrcR*, *hrpE1* and *hrpF* genes, which are encoded within the *hrpC*/*D*/*E*/*F* operons, but does not affect the expression of the *hrcC* and *hrcT* genes, which are encoded in the *hrpA*/*B* operons. This suggests that the expression of the *hrpC*/*D*/*E*/*F* operons, but not expression of the *hrpA*/*B* operons, is under the control of the ColRS system of *X. oryzae* pv. *oryzae*. In a previous study, Zhang *et al*. (2008) have shown that the expression of *hrpC*/*E* operons is affected in *colR/colS* mutants of *X. campestris* pv. *campestris*. In addition, Yan and Wang (2011) have shown that the expression of *hrpD6* and *hpaF* genes is affected in *colR*/*colS* mutants of *X. citri* ssp. *citri*.

Mutations in the *colR*/*colS* orthologues of *X. citri* ssp. *citri* affect biofilm formation, T3SS activity, catalase activity and lipopolysaccharide (LPS) production (Yan and Wang, 2011). These mutants also exhibit enhanced susceptibility to various environmental stresses, such as hydrogen peroxide, phenol and copper. These phenotypes may be attributable to defects in catalase activity and LPS production. Enhanced susceptibility to environmental stresses, such as cadmium, sodium chloride, phenol and antibiotics, has also been reported in *colR*/*colS* mutants of *X. campestris* pv. *campestris* (Zhang *et al*., 2008). Mutations in the *colR*/*colS* genes of *X. campestris* pv. *campestris* and *X. citri* ssp. *citri* (Qian *et al*., 2008; Yan and Wang, 2011; Zhang *et al*., 2008) cause virulence deficiency. These earlier reports and the results of this study demonstrate that the ColRS system is required for virulence of xanthomonads, because, either directly or indirectly, it serves as a master regulator of a number of virulence-associated functions.

The ColRS system of the plant growth-promoting *P. fluorescens* bacterial strain WCS365 has been shown to be important for root colonization (Dekkers *et al*., 1998). The promoter of the *colS* gene of *P. putida* has been shown to be rhizosphere induced (Ramos-Gonzalez *et al*., 2005), indicating its importance for root colonization. In *P. putida*, the ColRS system promotes tolerance to phenol, resistance to multiple metals and accumulation of Tn*4652* events that promote the utilization of phenol under starvation conditions (Horak *et al*., 2004; Hu and Zhao, 2007; Kivistik *et al*., 2006). It has been suggested that tolerance to phenol and resistance to metals are promoted by an effect on membrane functionality. The observed deficiencies in the expression of the genes involved in iron transport and T3SS suggest that, in xanthomonads, as well as in pseudomonads, the ColRS system is involved in the regulation of expression of proteins that function in membranes.

Insertions in the *colS* gene were found in only four of the 25 characterized *sop* mutants, with the remaining mutants having Tn*5* insertions in/close to five other genes. Fifteen *sop* mutants contained insertions in the XOO0007 gene, which encodes a conserved hypothetical protein and is immediately upstream of the *tonB* gene. Mutations in this gene affect growth on iron-limiting medium, but do not cause virulence deficiency. The TonB protein is essential for siderophore-mediated iron uptake (Postle and Kadner, 2003), and it is possible that insertions in the XOO0007 gene affect siderophore-mediated iron uptake either because the XOO0007 protein functions in this process or because of a polar effect of an insertion in this gene on *tonB* expression. Mutations in the *tonB* gene of *X. campestris* pv. *campestris* could be isolated only on ferrous sulphate-supplemented medium (Wiggerich *et al*., 1997). The absence of *tonB* insertions amongst the mutants isolated in our screen may be a result of the fact that, when performing the screen, bacterial cells were grown on medium that was not supplemented with ferrous sulphate. It is possible that *tonB* expression is driven by two promoters, one of which is upstream of XOO0007, whereas the other is in the 154-bp intergenic region between XOO0007 and *tonB*. Therefore, insertions in XOO0007 may only result in reduced expression of *tonB*. A defect in siderophore-mediated iron uptake has been shown previously to affect growth on iron-limiting medium without causing any virulence deficiency (Pandey and Sonti, 2010).

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The other *sop* mutants carried insertions in the following genes: two each in the *acnB* (XOO2862; encodes an Acn) and XOO1806 (encodes a conserved hypothetical protein) genes; one each in the *prpR* and *prpB* genes. The *prpR* and *prpB* genes are predicted to encode an activator of genes involved in propionate catabolism and an enzyme involved in propionate catabolism, respectively (Palacios and Escalante-Semerena, 2004). All of these mutants exhibit a deficiency for virulence and growth on iron-limiting medium. Insertions in these genes have not been shown previously to be involved in the promotion of virulence in any xanthomonad. Although polar effects cannot be ruled out without complementation data, a scrutiny of the downstream genes indicates that these have also not been shown to be involved in the virulence of xanthomonads.

AcnB has also been shown to function in the post-transcriptional regulation of gene expression. Studies in *Escherichia coli* indicate that the regulatory and enzymatic functions of AcnB are mutually exclusive. Under low-iron conditions, the dimeric form of AcnB switches to a catalytically inactive monomeric form and binds to target mRNAs to regulate their expression (Tang *et al*., 2005). It is possible that, in *X. oryzae* pv. *oryzae*, the correct expression of genes regulated by AcnB is required for the promotion of growth on iron-limiting medium and virulence.

Although mutations in several novel genes were isolated in our screen, no mutations were isolated in some genes that would be expected to result in siderophore overproduction. These include the *fur*, *feoB* and *xsuA* (which encodes siderophore receptor) genes. The *fur* mutants grow very poorly on PS medium (Subramoni and Sonti, 2005) and were probably excluded from our screen, because we only picked mutants that did not show an obvious growth defect. Although *feoB* mutants grow better than *fur* mutants in laboratory medium, they exhibit a growth defect on medium that is not supplemented with exogenous iron (Pandey and Sonti, 2010). This explains the absence of *feoB* insertions, which have been shown to overproduce siderophores, amongst the *sop* mutants. The mTn*5* element that was used for mutagenesis in this study causes polar mutations. An insertion in the *xsuA* gene would be polar on downstream *xss* genes that are required for siderophore production. Therefore, a mTn*5* insertion in the *xsuA* gene would result in a loss of siderophore production and not siderophore overproduction.

In summary, the results presented here indicate that *colR* and *colS* mutants of *X. oryzae* pv. *oryzae* exhibit reduced levels of expression of *hrp* genes and the *feoB* gene, together with concomitant deficiencies in T3SS activity and growth on iron-limiting medium. The virulence deficiency associated with the *colR* and *colS* mutants could be a result of defects in either T3SS functioning or iron uptake. The *acnB*, *prpR*, *prpB* and XOO1806 mutants exhibit deficiencies for virulence and growth on iron-limiting medium. It is possible that these mutants could also be affected in certain other functions, in addition to iron uptake/metabolism, that affect virulence. Further studies are needed to understand how these genes, as well as *colR*/*colS*, affect virulence and iron uptake/metabolism in *X. oryzae* pv. *oryzae*.

EXPERIMENTAL PROCEDURES

Bacterial strains, plasmids, primers and culture media

The bacterial strains and plasmids used are listed in Table 3. The primers used in this work are listed in Table S1. *Xanthomonas oryzae* pv. *oryzae* strains were grown at 28 °C in PS medium, and *E. coli* strains were grown at 37 °C in Luria Bertoni (LB) medium, as described previously (Pandey and Sonti, 2010). The concentrations of antibiotics used in this study were as follows: rifampicin (Rf), 50 μg/mL; ampicillin (Ap), 100 μg/mL; spectinomycin (Sp), 50 μg/mL; Km, 50 μg/mL for *E. coli* and 15 μg/mL for *X. oryzae* pv. *oryzae*. In addition, cycloheximide (80 μg/mL) was used to reduce fungal contamination. 2,2′-Dipyridyl (DP), an iron chelator, was prepared as a 10-mM stock solution and used at the concentrations indicated for each experiment.

Generation of mTn*5* mutant library of *X. oryzae* pv. *oryzae*

In vivo mutagenesis was performed by setting up biparental matings between donor *E. coli* (TP0051) containing the transposon mTn*5gusA40* (Sp resistance; transcription fusions with *gus* gene) on a suicide plasmid (pUT::Tn*5gusA40*) and recipient *X. oryzae* pv. *oryzae* strain BXO43. Biparental matings were performed as described by Dharmapuri and Sonti (1999). Briefly, late log-phase cultures of *X. oryzae* pv. *oryzae* were grown in 200 mL of PS at 28 °C. Cells were pelleted, washed and resuspended in 2 mL of sterile distilled water; 40 μL of this cell suspension was spotted onto Hybond N Plus nylon membrane (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK) that was overlaid on nutrient agar (Difco Laboratories, Detroit, MI, USA) and incubated at 28 °C for 2 h. An overnight culture of donor *E. coli* strain TP0051 was pelleted, washed and resuspended in water; 5 μL of this cell suspension [density of 10^7 colony-forming units (cfu)/mL] was mixed with recipient cells on the nylon membrane. The plates were incubated at 28 °C for 48 h, after which the cells were scraped from the plate and resuspended in 100 μL of sterile water. The cell suspension was plated on PSA plates containing Rf, Sp and cycloheximide. The plates were incubated at 28 °C and exconjugants were found to grow by 6–7 days. The *X. oryzae* pv. *oryzae* mTn*5* mutants were then patched onto PSA + Ap and PSA + Sp plates separately to eliminate any Ap^r colonies which might have arisen as a result of integration of pUT::Tn*5gusA40*. Only Sp^r, Ap^s colonies of *X. oryzae* pv. *oryzae* were selected for further study.

Screening for *X. oryzae* pv. *oryzae* mutants that overproduce siderophores

PSA plates supplemented with chrome azurol sulphonate (CAS) dye were prepared for the detection of siderophores, as described previously (Chatterjee and Sonti, 2002). Under iron-limiting conditions (caused by the addition of DP), siderophore production is visible as a yellow–orange zone

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Table 3 Bacterial strains and plasmids used in this study.

Strain/plasmid	Relevant characteristics*	Reference/source
Escherichia coli strains		
DH5α	F′/*endA*1 *hsdR17(rk$^-$ mk$^+$)supE44 thi-1 recA1 gyrA relA1*φ80dlacZΔM15Δ(*lacZYA-argF*)U169	Invitrogen
S17-1	RP4-2Tc::Mu-Km::Tn*7 pro hsdR recA* Tra$^+$, used as a mobilizing strain	Simon *et al*. (1983)
TP0051	λ pir derivative of S17-1, pUT::mTn*5gusA40*, Ap^r, Sp^r	Wilson *et al*. (1995)
Plasmids		
pK18mob	pUC18 derivative, Mob$^+$; Km^r, does not replicate in *X. oryzae* pv. *oryzae*	Schafer *et al*. (1994)
pHM1	Broad host range cosmid vector (~13.3 kb); Sp^r	Innes *et al*. (1988)
pSS2	pK18*mob* + 374-bp internal fragment of *colR* cloned into *Sma*I site of pK18*mob*	This work
pSS3	pK18*mob* + 739-bp internal fragment of *colS* cloned into *Sma*I site of pK18*mob*	This work
pAP24	pHM1 + 853-bp fragment containing full-length *colR* cloned into *Hind*III and *Sac*I sites of pHM1	This work
pAP25	pHM1 + 1462-bp fragment containing full-length *colS* cloned into *Hind*III and *Sac*I sites of pHM1	This work
Xanthomonas oryzae pv. *oryzae* strains		
BXO1	Wild-type; Indian isolate	Laboratory collection
BXO43	*rif-2*; derivative of BXO1	Laboratory collection
BXO2012	*hrpB6*::*bla rif*-2; HR$^-$, Ap^r, derived from BXO43	Jha *et al*. (2007)
BXO1843	*colR1*::pK18 *mob rif*-2; Km^r, derived from BXO43	This work
BXO1844	*colS5*::pK18 *mob rif*-2; Km^r, derived from BXO43	This work
BXO1845†	*colR$^+$ rif*-2; Km^s, revertant of BXO1843	This work
BXO1846†	*colS$^+$ rif*-2, Km^s, revertant of BXO1844	This work
BXO2241	*colS5*::pK18*mob rif-2*/pAP25; ColS$^+$, Km^r, Sp^r	This work
BXO2243	*colR1*::pK18*mob rif-2*/pAP24; ColR$^+$, Km^r, Sp^r	This work
sop mutants‡		
BXO1818	*colS1*::mTn*5 rif*-2	This work
BXO1823	*colS2*::mTn*5 rif*-2	This work
BXO1824	*colS3*::mTn*5 rif*-2	This work
BXO1835	*colS4*::mTn*5 rif*-2	This work
BXO1820	*XOO1806-1*::mTn*5 rif*-2	This work
BXO1834	*XOO1806-2*::mTn*5 rif*-2	This work
BXO1822	*acnB1*::mTn*5 rif*-2	This work
BXO1833	*acnB2*::mTn*5 rif*-2	This work
BXO1805	*XOO0007-1*::mTn*5 rif*-2	This work
BXO1807	*XOO0007-2*::mTn*5 rif*-2	This work
BXO1812	*XOO0007-3*::mTn*5 rif*-2	This work
BXO1813	*zxx301*::mTn*5 rif*-2; an insertion in XOO0007–XOO0008 intergenic region	This work
BXO1814	*XOO0007-4*::mTn*5 rif*-2	This work
BXO1815	*zxx302*::mTn*5 rif*-2; an insertion in XOO0007–XOO0008 intergenic region	This work
BXO1821	*XOO0007-5*::mTn*5 rif*-2	This work
BXO1826	*XOO0007-6*::mTn*5 rif*-2	This work
BXO1827	*XOO0007-7*::mTn*5 rif*-2	This work
BXO1830	*XOO0007-8*::mTn*5 rif*-2	This work
BXO1831	*XOO0007-9*::mTn*5 rif*-2	This work
BXO1832	*XOO0007-10*::mTn*5 rif*-2	This work
BXO1837	*XOO0007-11*::mTn*5 rif*-2	This work
BXO1839	*XOO0007-12*::mTn*5 rif*-2	This work
BXO1840	*XOO0007-13*::mTn*5 rif*-2	This work
BXO1816	*prpR1*::mTn*5 rif*-2	This work
BXO1836	*prpB1*::mTn*5 rif*-2	This work

**rif-2* indicates a mutation that confers resistance to rifampicin. HR$^-$ indicates deficiency for hypersensitive response. Ap^r, Sp^r and Km^r indicate resistance to ampicillin, spectinomycin and kanamycin, respectively. Km^s indicates kanamycin sensitivity. *zxx* indicates mutations that are in the intergenic region between XOO0007 and XOO0008 (*tonB*). Gene numbers are as specified by Lee *et al*. (2005).

†These strains were isolated as virulence-proficient revertants from rice leaves inoculated with *X. oryzae* pv. *oryzae* strains BXO1843 and BXO1844.

‡*sop* indicates siderophore overproduction phenotype.

around the bacterial colony. PSA-CAS plates are apparently rich in iron as wild-type *X. oryzae* pv. *oryzae* does not secrete siderophores in this condition. Mutants were patched with a toothpick on PSA-CAS plates and the siderophore production phenotype was assessed after 24 h of incubation at 28 °C. Three rounds of screening were performed with selected *sop* mutants following the same protocol to eliminate mutants with inconsistent phenotypes.

Southern hybridization

Genomic DNA was isolated from *X. oryzae* pv. *oryzae* strains by the protocol described previously (Leach *et al*., 1990). Restriction digestion of DNA and α-^{32}P-dATP labelling were performed as described by Sambrook *et al*. (1989). Genomic DNA was digested with *Kpn*I (NEB, Beverly, NE, USA) because it does not cleave within the mTn*5* sequence (Wilson *et al*.,

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1995). Southern hybridization was performed according to the protocol described by Yashitola *et al*. (1997). The plasmid pUT::Tn*5gusA40* isolated from *E. coli* strain TP0051 was used as a probe.

TAIL PCR

TAIL PCR was performed according to the protocol described by Liu and Whittier (1995). The primers used for TAIL PCR are indicated in Table S1. Briefly, *X. oryzae* pv. *oryzae* colonies were picked with a toothpick, resuspended in 100 μL of sterile water and lysis was carried out at 94 °C for 15 min. Primary PCR was performed with 0.2 μM mTn*5*-specific primer and 3 μM arbitrary primer (AD). Secondary and tertiary PCRs were performed with a 10-fold lower concentration of deoxynucleoside triphosphates (dNTPs). PCR cycling conditions were similar to those described by Liu and Whittier (1995). The annealing temperatures for both primary and secondary PCRs were kept at 55 °C. For the tertiary reaction, the annealing temperature was 36 °C. The secondary and tertiary PCR samples were analysed on a 1.5% agarose gel, and a band shift corresponding to a difference in the product sizes of secondary and tertiary PCRs (150 bp with *gus* side primers and 100 bp with *spec* side primers of the mTn*5* element) was taken as an indication of the presence of a specific product.

DNA sequencing and analysis

DNA sequencing of PCR-amplified fragments was performed using the primers directed outwardly from the *gus* and *spec* genes of the mTn*5* element (Wilson *et al*., 1995). The sequencing reactions and detection were performed using an ABI Prism Automated Sequencer 3730 (Perkin Elmer, Foster City, CA, USA). Homology and conserved domain searches were performed in the National Center for Biotechnology Information (NCBI) database using the BLAST algorithm (Altschul *et al*., 1990).

Generation and complementation of *colR* and *colS* mutants

The *colR* and *colS* genes were each disrupted by integration of the suicide vector pK18*mob* (Schafer *et al*., 1994) into chromosomal DNA via homologous recombination. The internal fragments of *colR* (374 bp) and *colS* (739 bp) were generated by PCR using genomic DNA of *X. oryzae* pv. *oryzae* strain BXO43 as a template with the following respective primer sets: colRIP1/colRIP2 and colSF1/colSR1 (Table S1). These fragments were cloned into pK18*mob* digested with *Sma*I, and the plasmids obtained were designated as pSS2 and pSS3, respectively. These constructs were then transferred from *E. coli* strain S17-1 (Simon *et al*., 1983) to *X. oryzae* pv. *oryzae* via biparental mating, and *colR* (BXO1843) and *colS* (BXO1844) mutants were selected on Km-containing medium. PCR was performed with the flanking primers (colRPF1/colRPR1 and colSGF1/colSGR1) in combination with vector-specific primers (M13F and M13R), and the fragments obtained were sequenced to confirm the disruption of *colR* and *colS* genes in the mutants.

For complementation, 853- and 1462-bp DNA fragments containing full-length *colR* and *colS* genes, respectively, were amplified by PCR using genomic DNA of *X. oryzae* pv. *oryzae* and the primer sets ColRCF/ColRCR and ColSCF/ColSCR. The amplified fragments were individually cloned as *Hind*III-*Sac*I fragments into the broad host range vector pHM1 (Innes *et al*., 1988) to create the recombinant plasmids: pAP24 (for *colR*) and pAP25 (for *colS*). The pAP24 and pAP25 plasmids were introduced into the *colR* (BXO1843) and *colS* (BXO1844) mutants, respectively, to obtain the complemented strains: BXO2243 and BXO2241.

Assays for virulence, HR and callose deposition

Xanthomonas oryzae pv. *oryzae* strains were grown to saturation and inoculated on 30–40-day-old glasshouse-grown rice plants of the highly susceptible cultivar Taichung Native (TN-1). Inoculation was performed by dipping scissors into bacterial cultures and clipping the tips of rice leaves (Kauffman *et al*., 1973). Lesion lengths were measured 7 and 14 days after inoculation and expressed as the mean lesion length ± standard deviation. For HR assays, leaves of tomato plants were infiltrated with *X. oryzae* pv. *oryzae* strains (10^8 cfu/mL) using a blunt-ended plastic syringe. HR symptoms (browning of the infiltrated area) were observed 16 h after infiltration. For callose assays, rice leaves were infiltrated with *X. oryzae* pv. *oryzae* strains (10^8 cfu/mL) using a blunt-ended plastic syringe. After 16 h, the leaves were cleared with 100% ethanol, rehydrated with 50% ethanol and stained with aniline blue. The leaf samples were then mounted on a slide in 50% glycerol, and analysed by an Axio Imager Z1 (Carl Zeiss, Oberkochen, Germany) microscope using a 4′,6-diamidino-2-phenylindole (DAPI) fluorescence filter.

Re-isolation of bacteria from rice leaves and revertant identification

Infected leaves were surface sterilized by dipping in 1% (v/v) sodium hypochlorite (Loba Chemie, Mumbai, India) for 1 min and washing three times in distilled water. The leaves were cut at the leading edge of the lesion and dipped in 1 mL of sterile water for 5 min. Bacteria that exuded from the cut edges of the leaves were isolated by plating for individual colonies on PSA. These colonies were patched on PSA + Km plates and the $ColR^+$ (BXO1845) and $ColS^+$ (BXO1846) revertants were identified as Km-sensitive colonies. The Km-sensitive colonies were patched on PSA-CAS plates to assess the ability to produce siderophores. In addition, revertants were checked for their ability to cause disease and to grow on iron-limiting medium.

Growth experiments

Xanthomonas oryzae pv. *oryzae* strains were grown to log-phase in PS medium at 28 °C to obtain pre-inoculum. For each strain, 3 mL PS and 3 mL PS + 140 μM DP were inoculated with 0.1% pre-inoculum. Cultures were dilution plated on PSA plates immediately (0 h) and 48 h after inoculation to determine cfu/mL. The bacterial count was expressed as the log value of cfu/mL.

RNA isolation and quantitative RT-PCR

To investigate the effect of *colR*/*colS* genes on the expression of *hrp*, *xssE* and *feoB* genes, real-time quantitative PCR was carried out. *Xanthomonas*

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oryzae pv. *oryzae* strains were grown in PS medium to an optical density at 600 nm (OD_{600}) of 1.0. These cultures were used to isolate RNA and to examine the expression of *xssE* and *feoB* genes; in contrast, for *hrp* genes, cultures incubated in *hrp*-inducing XOM2 medium (Tsuge *et al*., 2002) were used for total RNA isolation. For incubation in XOM2 medium, PS-grown cultures were centrifuged and the pellets were washed three times with MilliQ water (Millipore, Billerica, MA, USA), and then transferred to XOM2 medium. Cultures were further incubated for 18 h, and total RNA from each of the samples was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The total RNA concentration was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA).

RNA samples were treated with RNase-free DNaseI (NEB) to remove any DNA contamination. A total of 1 μg of RNA was reverse transcribed by SuperScript III reverse transcriptase enzyme (Invitrogen) using 50 ng of random hexamers, according to the supplier's instructions. One microlitre of the 1:10 diluted cDNA was subjected to real-time quantitative PCR using SYBR GreenER qPCR supermix (Invitrogen) and gene-specific primers designed to amplify 100–155-bp fragments from each gene of interest and the gene encoding for 16S ribosomal RNA. The primers (Table S1) were designed to amplify the fragments from *hrcC*, *hrcT*, *hrcU*, *hrcR*, *hrpE1*, *hrpF*, *xssE* and *feoB* genes of *X. oryzae* pv. *oryzae*. After 10 min of initial denaturation at 95 °C, the samples were subjected to cycling parameters of 95 °C for 15 s, 58 °C for 30 s and 72 °C for 30 s (40 cycles) using a 7900 HT sequence detection system (Applied Biosystems, Foster City, CA, USA). The relative expression of *hrp* genes was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) with *16S rRNA* as an endogenous control.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Location of mTn*5* insertions and gene organization in the *Xanthomonas oryzae* pv. *oryzae* genomic region containing the XOO0007 gene. The filled triangles represent the locations of the mTn*5* insertions. The arrows indicate the transcriptional orientations of the genes. The bold lines indicate open reading frames (ORFs) and the lighter lines indicate intergenic regions. Several *sop* mutants carry an mTn*5* insertion at the same site in the XOO0007 gene. These include *XOO0007-4* (BXO1814), *XOO0007-6* (BXO1826) and *XOO0007-7* (BXO1827); *XOO0007-1* (BXO1805) and *XOO0007-9* (BXO1831); and *XOO0007-8* (BXO1830) and *XOO0007-11* (BXO1837). The *zxx301* (BXO1813) and *zxx302* (BXO1815) insertions are in the intergenic region between the XOO0007 and *tonB* genes, which are in the same transcriptional orientation. The gene organization given here is as indicated for the genome of *X. oryzae* pv. *oryzae* strain KACC10331 (Lee *et al*., 2005).

Table S1 List of oligonucleotide primers used in this study.

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