Molecular Plant Pathology

MOLECULAR PLANT PATHOLOGY (2011) **12**(3), 227–237 DOI: 10.1111/J.1364-3703.2010.00661.X

The *rsmA*-like gene *rsmA*$_{Xoo}$ of *Xanthomonas oryzae* pv. *oryzae* regulates bacterial virulence and production of diffusible signal factor

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SUMMARY

The plant-pathogenic prokaryote *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) causes bacterial leaf blight, one of the most destructive diseases of rice. A nonpolar mutant of the *rsmA*-like gene *rsmA*$_{Xoo}$ of the *Xoo* Chinese strain 13751 was constructed by homologous integration with a suicide plasmid. Virulence tests on a host plant, namely the hybrid rice cultivar Teyou 63, showed that the mutant had lost its virulence almost completely, whereas tests on a nonhost, namely castor-oil plant (*Ricinus communis*), showed that the mutant had also lost the ability to induce a hypersensitive response in the nonhost. In addition, the *rsmA*$_{Xoo}$ mutant produced significantly smaller amounts of the diffusible signal factor, extracellular endoglucanase, amylase and extracellular polysaccharide, but showed significantly higher glycogen accumulation, bacterial aggregation and cell adhesion. The expression of most *hrp* genes, genes encoding AvrBs3/PthA family members, *rpfB*, *xrvA*, *glgA*, *eglXoB* and *XOO0175* (encoding an α-amylase) was down-regulated in the *rsmA*$_{Xoo}$ mutant. All phenotypes and expression levels of the tested genes in the *rsmA*$_{Xoo}$ mutant were restored to their levels in the wild-type by the presence of *rsmA*$_{Xoo}$ *in trans*. These results indicate that *rsmA*$_{Xoo}$ is essential for the virulence of *Xoo*.

INTRODUCTION

The Gram-negative bacterium *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) causes bacterial leaf blight, one of the most destructive diseases of rice, which limits the production of this staple food (Mew *et al*., 1993). *Xoo* typically invades the plant through hydathodes or wounds along the leaf margins, reaches the xylem vessels and multiplies in the intercellular spaces to cause a vascular disease, resulting in tan-grey to white lesions along the leaf veins (Nino-Liu *et al*., 2006).

Many Gram-negative plant-pathogenic bacteria rely on the type III secretion system (T3SS) to inject effectors into the host cell, which leads to either disease or a resistant reaction (Grant *et al*., 2006). T3SS of *Xanthomonas*, which is encoded by a hypersensitive response and pathogenicity (*hrp*) gene cluster, is necessary for pathogenicity in susceptible hosts and for a hypersensitive response (HR) in resistant plants and nonhost plants. T3SS may also play a role in the secretion of other virulence factors (Büttner and Bonas, 2010; Büttner and He, 2009). In *Xoo*, the *hrp* gene cluster consists of the conserved *hrp* region, which comprises *hpa1*, *hpa2*, *hrpA* and *hrp* operons *hrpB–E*, and the variable region, which comprises *hpaC*, *hpaD*, *hrpF* and *hpaF* (Ochiai *et al*., 2005). HrpG, which is the OmpR family regulator, and HrpX, which is the AraC-type regulator, are the two key regulators of *hrp* genes in *Xanthomonas*. The expression of *hrpX* is up-regulated by HrpG, which is activated in response to as yet unknown signals (Kamdar *et al*., 1993; Koebnik *et al*., 2006; Wengelnik and Bonas, 1996; Wengelnik *et al*., 1996).

Most avirulence (Avr) proteins that activate a plant's defence responses are type III effectors in phytopathogenic bacteria (Grant *et al*., 2006; Mansfield, 2009). Several *avr* genes have been cloned and identified in *Xoo* (Lee *et al*., 2009; White and Yang, 2009), and their encoded products, except Ax21 (formerly AvrXa21; Lee *et al*., 2009), belong to the AvrBs3/PthA family (White and Yang, 2009; White *et al*., 2000). The *avr* gene *avrXa7* is also a determinant of virulence in *Xoo* (White and Yang, 2009). Other type III effectors belonging to the AvrBs3/PthA family and encoded by the genes *pthXo1*, *pthXo2*, *pthXo3*, *pthXo6* and *pthXo7* from *Xoo* are known to contribute to bacterial virulence under appropriate conditions (White and Yang, 2009).

The virulence of *Xanthomonas campestris* pv. *campestris* (*Xcc*) also depends on cell–cell signalling mediated by the small diffusible signal factor (DSF) and by the RpfC/RpfG two-component regulatory system, which couples DSF sensing to the intracellular

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regulatory networks through a second messenger, namely cyclic diguanylate (di-GMP), and a global regulator, namely Clp (Dow, 2008; Fouhy *et al*., 2006; He and Zhang, 2008). DSF was first identified in *Xcc* (Barber *et al*., 1997), and was structurally identified as *cis*-11-methyl-2-dodecenoic acid (Wang *et al*., 2004). The factor acts as a cell–cell communication signal involved in the expression of virulence genes and dispersal of the biofilm to control the production of extracellular enzymes and extracellular polysaccharide (EPS), and mediates the synthesis of the flagellum, resistance to toxins and oxidative stress, and aerobic respiration (He *et al*., 2006). Two genes, *rpfF* (encoding a putative enoyl CoA hydratase) and *rpfB* (encoding a long-chain fatty acyl CoA ligase), have been implicated in the synthesis of DSF (Barber *et al*., 1997). In *Xoo*, mutations in *rpfF* make the mutants incapable of producing DSF (Chatterjee and Sonti, 2002; He *et al*., 2010).

Virulence factors, including cellulases, xylanases, pectinases and proteases, which are secreted by plant-pathogenic bacteria, can break down host cell walls and may play an important role in virulence (Büttner and Bonas, 2010). Extracellular endoglucanase is involved in the virulence of *Xcc* and *Xoo* (Büttner and Bonas, 2010; Gough *et al*., 1988; Hu *et al*., 2007; Schröter *et al*., 2001). Some studies have demonstrated that extracellular xylanase, cellobiosidase and esterase are important to the virulence of *Xoo* (Aparna *et al*., 2009; Jha *et al*., 2007; Rajeshwari *et al*., 2005; Ray *et al*., 2000). EPS is another important virulence factor in *Xanthomonas* (Büttner and Bonas, 2010). In *Xoo*, the *gum* cluster is involved in EPS synthesis (Dharmapuri and Sonti, 1999; Kim *et al*., 2009; Rajeshwari and Sonti, 2000), and *rpfC* plays an important role in regulating EPS synthesis (Tang *et al*., 1996).

The bacterial RNA-binding protein RsmA (repressor of secondary metabolism) is a global post-transcriptional regulator, which was discovered as a potent repressor of glycogen synthesis in *Escherichia coli* and designated as CsrA (carbon storage regulator) (Romeo, 1998). The CsrA of *E. coli* was further found to repress gluconeogenesis, peptide transport and biofilm formation, and to activate glycolysis, acetate metabolism and cell motility (Lapouge *et al*., 2008; Romeo, 1998). In addition, RsmA/CsrA was found to be involved in the pathogenesis of several bacterial pathogens in animals and plants (Chao *et al*., 2008; Fields and Thompson, 2008; Lapouge *et al*., 2008; Lucchetti-Miganeh *et al*., 2008).

RsmA/CsrA of bacterial pathogens can regulate the expression of virulence genes negatively or positively. For example, CsrA of *Salmonella enterica* serovar Typhimurium positively regulates the expression of invasion genes and of the components of T3SS associated with virulence (Lucchetti-Miganeh *et al*., 2008). However, in *Erwinia carotovora*, a plant pathogen, RsmA represses the expression of some T3SS-related genes and also destabilizes mRNA transcripts encoding enzymes that degrade cell walls in plants, including cellulase, pectate lyase and protease (Mole *et al*., 2007). In the plant-pathogenic bacterium *Xcc* strain 8004, the deletion of $rsmA_{Xcc}$ leads to a complete loss of virulence towards the host plant Chinese radish, HR in the nonhost pepper (cultivar ECW-10R) and motility on agar plates. Furthermore, a mutation in $rsmA_{Xcc}$ reduces significantly the production of extracellular amylase, endoglucanase and polysaccharide, but increases significantly the accumulation of intracellular glycogen, bacterial aggregation and cell adhesion. The mutation also results in significantly reduced expression of genes encoding T3SS, type III effectors and the bacterial aggregate-dispersing enzyme endo-β-1,4-mannanase (Chao *et al*., 2008).

Whole-genome sequencing has revealed that *rsmA/csrA* homologues exist in *Xoo* (Lee *et al*., 2005; Ochiai *et al*., 2005; Salzberg *et al*., 2008), but little is known about their role in the pathogenesis of *Xoo*. In this article, we present genetic evidence to demonstrate that $rsmA_{Xoo}$, the *rsmA/csrA* homologue in *Xoo*, acts as an important regulator of virulence and DSF production.

RESULTS

$rsmA_{Xoo}$ is essential for the virulence and HR of *Xoo*

The *rsmA*-like gene $rsmA_{Xoo}$ (accession number HM988729), an open reading frame (ORF) of *Xoo* Chinese strain 13751 (Tang *et al*., 1996) (Table 1), is 213 bp long and encodes a protein comprising 70 amino acids. The $rsmA_{Xoo}$ gene was identical to *XOO_2790* (accession number NC_007705.1), *XOO2938* (accession number NC_006834.1) and *PXO_00146* (accession number NC_010717.1), all of which were annotated as *csrA*/*rsmA* (hereafter $rsmA_{Xoo}$) in the sequenced genome of the *Xoo* strains MAFF311018, KACC10331 and PXO99^{A}, respectively. $RsmA_{Xoo}$ displays 74% identity with *E. coli* K-12 CsrA (NP_417176.1), 93% with *Xcc* 8004 $RsmA_{Xcc}$ (YP_243576.1) and 100% with *X. axonopodis* pv. *citrus* 306 (*Xac*) $RsmA_{Xac}$ (NP_642074.1) and *X. campestris* pv. *vesicatoria* 85-10 (*Xcv*) $RsmA_{Xcv}$ (YP_363507.1). The nearest gene upstream of $rsmA_{Xoo}$ was *alaS* (encoding a putative alanyl-tRNA synthetase), whereas the nearest gene downstream was *tRNA-Ser* (encoding tRNA-Ser) (Fig. S1, see Supporting Information). Sequence analyses showed that no other gene in the sequenced genome of *Xoo* strains shared significant homology with $rsmA_{Xoo}$.

To study the potential biological function of $rsmA_{Xoo}$, a nonpolar mutant of $rsmA_{Xoo}$, named GXN2790, was constructed in *Xoo* Chinese strain 13751 by homologous integration with the suicide plasmid pKMob18GII (Katzen *et al*., 1999) derivative $pKrsmA_{Xoo}$ (Table 1). Mutation was confirmed by complementation with the entire sole $rsmA_{Xoo}$ gene. The complemented strain GXC2790 was constructed by introducing the recombinant plasmid $pLrsmA_{Xoo}$ containing the entire wild-type $rsmA_{Xoo}$ gene into the mutant GXN2790 (Table 1).

Table 1 Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics*	Reference or source
Xanthomonas oryzae pv. *oryzae* strains		
13751	Wild-type; Chinese isolate, Sm^r	Tang *et al*. (1996)
GXN2790	As 13751, but *rsmA*$_{Xoo}$::pK18MobGII, Sm^r Km^r	This work
GXC2790	GXN2790 harbouring pL6rsmA$_{Xoo}$, Sm^r Km^r Tc^r	This work
13751hrcV	As 13751, but *hrcV*::Tn5, Sm^r Km^r	Feng *et al*. (2001)
Xanthomonas campestris pv. *campestris* strains		
DM2506	As wild-type *Xcc* 8004, but the coding region of *rsmA*$_{Xcc}$ (XC2506) has been deleted, Rif^r	Chao *et al*. (2008)
8523	As wild-type strain 8004, but *rpfF*::Tn5*lac*, Rif^r Km^r	Tang *et al*. (1991)
Plasmids		
pGEM-3Zf(+)	Harbouring multiple cloning site, Amp^r	Promega
pRK2073	Helper plasmid, Tra$^+$, Mob$^+$, ColE1; Spc^r	Leong *et al*. (1982)
pK18MobGII	Mob$^+$; ColE1 *gusA*, Km^r	Katzen *et al*. (1999)
pKrsmA$_{Xoo}$	pK18MobGII containing a 131-bp internal fragment of *rsmA*$_{Xoo}$ gene, Km^r	This work
pLAFR6	Broad host range cloning vector, Tc^r	Huynh *et al*. (1989)
pL6rsmA$_{Xoo}$	pLAFR6 containing a 703-bp fragment including the *rsmA*$_{Xoo}$ gene, Tc^r	This work

*Sm^r, Km^r, Tc^r, Spc^r and Rif^r indicate resistance to streptomycin, kanamycin, tetracycline, spectinomycin and rifampicin, respectively.

To determine whether mutations in *rsmA*$_{Xoo}$ affect the growth of *Xoo*, we examined the growth of the *rsmA*$_{Xoo}$ mutant strain GXN2790, the wild-type strain 13751 and the complemented strain GXC2790 in complete medium, namely OB (Tang *et al*., 1996), and in minimal medium, namely XOM2 (Tsuge *et al*., 2002). The growth of all three strains was comparable in both media ($P > 0.05$, *t*-test) (data not shown), indicating that *rsmA*$_{Xoo}$ is not required for the growth of *Xoo* in either of the two media.

The virulence of *rsmA*$_{Xoo}$, the nonpolar mutant GXN2790 and the complemented strain GXC2790 was tested on a hybrid rice cultivar, namely Teyou 63, by the leaf-clipping method (Kauffman *et al*., 1973), with the wild-type strain 13751 serving as the control. Fourteen days after inoculation with a final concentration of either 1×10^6 colony-forming units (cfu)/mL (data not shown) or 1×10^8 cfu/mL, symptoms of bacterial leaf blight of comparable intensity appeared along the leaves inoculated with either the wild-type or the complemented strain, with no difference in lesion length ($P > 0.05$, *t*-test). However, leaves inoculated with the mutant strain GXN2790 were almost free from lesions (Fig. 1a,b) ($P < 0.01$, *t*-test), indicating that *rsmA*$_{Xoo}$ is essential for the virulence of *Xoo*.

To investigate the role of *rsmA*$_{Xoo}$ in the growth of *Xoo* inside the host, we compared the populations of the *rsmA*$_{Xoo}$ mutant GXN2790, the wild-type strain 13751 and the complemented strain GXC2790 on inoculated rice leaves. The population of the mutant strain GXN2790 recovered from infected leaves was significantly lower than that of the wild-type and the complemented strain at each of the test points ($P < 0.01$, *t*-test) (Fig. 1c). There was no difference in growth between the complemented and wild-type strain ($P > 0.05$, *t*-test) (Fig. 1c). These results indicate that *rsmA*$_{Xoo}$ is required for the proliferation of *Xoo* in host plants.

To determine the role of *rsmA*$_{Xoo}$ in inducing an HR in castor-oil plant (*Ricinus communis*), a nonhost, its leaves were inoculated separately with the *rsmA*$_{Xoo}$ mutant GXN2790 and the *Xoo hrcV* mutant, a type III-deficient mutant that cannot elicit an HR in nonhost plants (Feng *et al*., 2001) (Table 1). The mutant GXN2790 was unable to trigger an HR, whereas the wild-type and the complemented strain GXC2790 were able to do so (Fig. 2). As expected, the negative control *hrcV* mutant elicited no visible HR (Fig. 2). Therefore, it is clear that *rsmA*$_{Xoo}$ is essential for *Xoo* to elicit an HR in nonhost castor-oil plant.

rsmA$_{Xoo}$ positively regulates DSF biosynthesis

The restored endoglucanase activity of *Xcc rpfF* mutant strain 8523 in the presence of DSF extracted from *Xoo* strains is positively correlated with the level of DSF produced by *Xoo* strains (Feng *et al*., 2009). The restored endoglucanase activity of strain 8523, added to DSF extracted from various *Xoo* strains, was assayed using strain 8523 as the control. DSF extracted from the *rsmA*$_{Xoo}$ mutant strain GXN2790 resulted in significantly less endoglucanase production than DSF extracted from the wild-type strain 13751 and the complemented strain GXC2790 ($P < 0.01$, *t*-test) (Fig. 3); however, the latter led to a comparable level of endoglucanase production in mutant 8523 ($P > 0.05$, *t*-test) (Fig. 3). These results indicate that *rsmA*$_{Xoo}$ positively regulates DSF production in *Xoo*.

rsmA$_{Xoo}$ regulates EPS production positively and glycogen accumulation negatively

EPS is an important factor in the virulence of *Xoo* (Büttner and Bonas, 2010; Dharmapuri *et al*., 2001; Tang *et al*., 1996). To

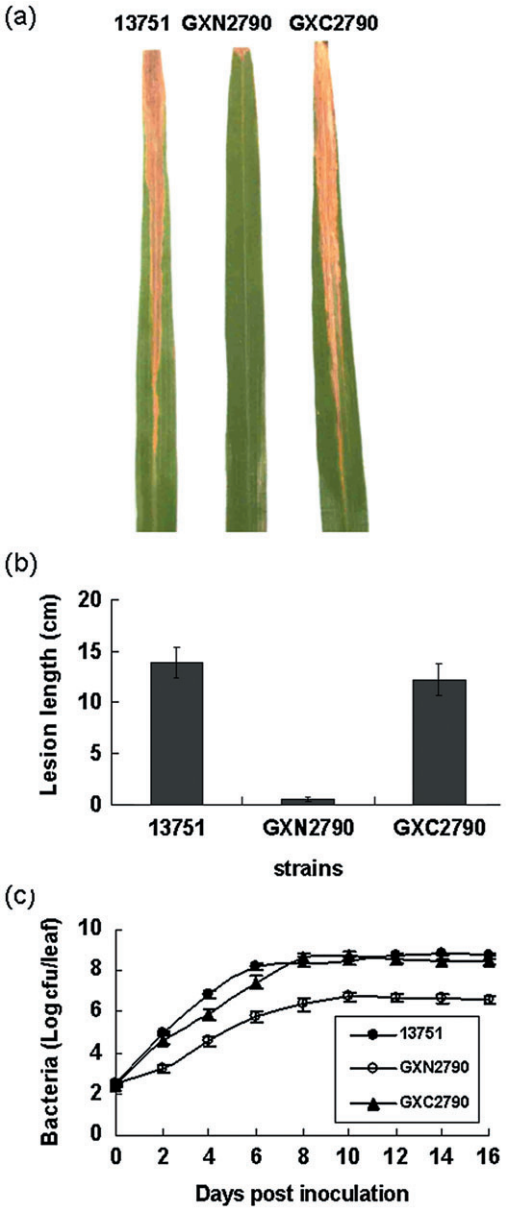

Fig. 1 *rsmA*$_{Xoo}$ is essential for the virulence of *Xanthomonas oryzae* pv. *oryzae*. (a) Rice leaf blight symptoms on leaves of the hybrid rice cultivar Teyou 63, 14 days after inoculation with *X. oryzae* pv. *oryzae* strains at an optical density at 600 nm (OD_{600}) of 0.1 [1×10^8 colony-forming units (cfu)/mL]. (b) Average length of lesions caused by *X. oryzae* pv. *oryzae* inoculated at $OD_{600} = 0.1$ (1×10^8 cfu/mL). Data are means ± standard deviation from three replications, each with 30 leaves. The results presented are from a representative experiment; similar results were obtained in the other two independent experiments. (c) Growth of bacteria in inoculated leaves of rice Teyou 63. Bacteria were inoculated by the leaf-clipping method at $OD_{600} = 0.1$ (1×10^8 cfu/mL). Three inoculated leaves for each strain were removed at 2-day intervals and homogenized in sterile water. The diluted homogenate was plated onto a rich medium supplemented with appropriate antibiotics. Bacterial cfu was counted after incubation at 28°C for 3 days, and was expressed as cfu per leaf. Data are the means ± standard deviation from three replications.

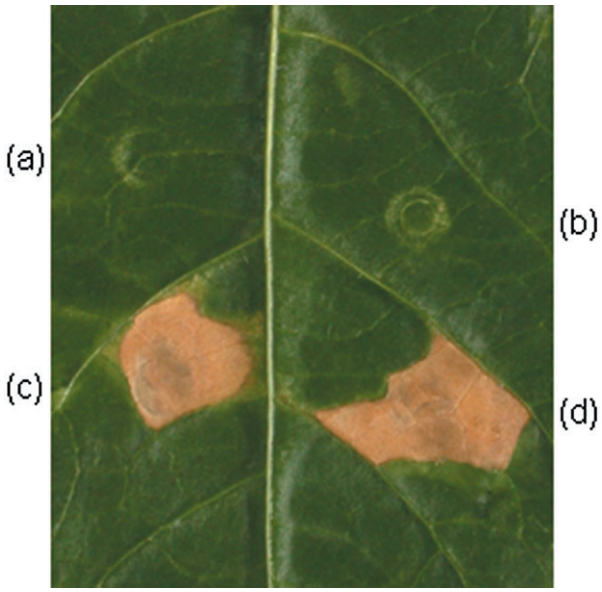

Fig. 2 Induction of the hypersensitive response (HR) on the leaves of a nonhost, namely castor-oil plant (*Ricinus communis*), by *Xanthomonas oryzae* pv. *oryzae* strains. Approximately 20 μL of bacterial culture suspended in 10 mM sodium phosphate buffer was infiltrated into leaf mesophyll tissue using a 1-mL blunt-end plastic syringe at an inoculum level of 5×10^8 colony-forming units (cfu)/mL. The photograph was taken 48 h after infiltration. Four replicates were maintained for each experiment, and each experiment was repeated three times. The results presented are from a representative experiment; similar results were obtained in all other independent experiments. (a) *rsmA*$_{Xoo}$ mutant strain GXN2790. (b) 13751 *hrcV* mutant (type III-deficient strain, negative control). (c) Complemented strain GXC2790. (d) Wild-type strain 13751.

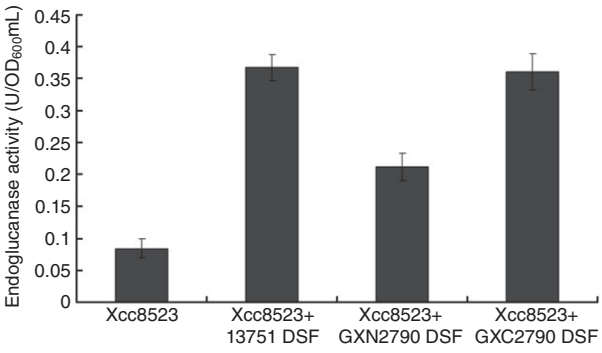

Fig. 3 *rsmA*$_{Xoo}$ regulates diffusible signal factor (DSF) biosynthesis in *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). DSF extracted from culture supernatants of different strains of *Xoo* was bioassayed by measuring the restoration of endoglucanase activity in the *X. campestris* pv. *campestris* (*Xcc*) *rpfF* mutant 8523. Three replicates were maintained for each experiment and each experiment was repeated three times. Endoglucanase activity is expressed as units per OD_{600} unit per millilitre. Data shown are the means ± standard deviation for one experiment; similar results were obtained in all experiments.

Table 2 Extracellular polysaccharide, intracellular glycogen, extracellular endoglucanase and amylase levels produced by *Xanthomonas oryzae* pv. *oryzae* strains.*

Strain	Extracellular polysaccharide (g/L)	Intracellular glycogen (g/L)	Extracellular endoglucanase (U/OD_{600}/mL)	Extracellular amylase (U/OD_{600}/mL)
13751(wild-type)	15.1 ± 1.56	2.9 ± 0.45	0.38 ± 0.02	0.19 ± 0.01
GXN2790	9.6 ± 1.04**	4.7 ± 0.49**	0.16 ± 0.03**	0.08 ± 0.01**
GXC2790	14.1 ± 1.45	2.6 ± 0.27	0.36 ± 0.04	0.17 ± 0.01

*Data are the means ± standard deviation from three replications. Statistically significant differences between the mutant GXN2790 and the wild-type strain 13751 are indicated by **($P < 0.01$). The experiment was repeated three times, and similar results were obtained each time.

determine whether a mutation in *rsmA*$_{Xoo}$ had any effect on EPS production, the EPS yields of the three *Xoo* strains were measured, and were found to be significantly less in the *rsmA*$_{Xoo}$ mutant strain GXN2790 ($P < 0.01$, *t*-test) and comparable in the complemented strain GXC2790 and the wild-type ($P > 0.05$, *t*-test) (Table 2). These data indicate that *rsmA*$_{Xoo}$ regulates EPS production positively in *Xoo*.

It is noteworthy that the *rsmA*$_{Xoo}$ mutant strain GXN2790 was stained dark brown on OB agar plates because of excess accumulation of glycogen, whereas the other two strains turned yellowish-brown when the production of intracellular polysaccharide glycogen was assessed by iodine vapour staining (Chao *et al*., 2008) (data not shown). The amount of glycogen in *Xoo* cells was measured by the method described by Feng *et al*. (2009). Notably, the complemented strain GXC2790 produced about the same amount of glycogen as the wild-type ($P > 0.05$, *t*-test), whereas the *rsmA*$_{Xoo}$ mutant strain GXN2790 produced almost twice as much (Table 2). The results indicate that *rsmA*$_{Xoo}$ negatively regulates intracellular glycogen accumulation in *Xoo*.

rsmA$_{Xoo}$ is involved in the production of extracellular endoglucanase and amylase

Extracellular enzymes, such as xylanase, endoglucanase, amylase and protease, contribute individually or collectively to the virulence of *Xanthomonas* (Chatterjee *et al*., 2003; Hu *et al*., 2007; Rajeshwari *et al*., 2005; Ray *et al*., 2000). To verify whether the virulence-impaired *rsmA*$_{Xoo}$ mutant exhibits any defects in the production of these enzymes that contribute to virulence, we measured the activity of these extracellular enzymes in the *rsmA*$_{Xoo}$ mutant GXN2790. The mutant displayed about as much xylanase and protease activity as the wild-type strain 13751 (data not shown), but significantly less endoglucanase and amylase activity ($P < 0.01$, *t*-test) (Table 2). In contrast, endoglucanase and amylase activities in the complemented strain GXC2790 and in the wild-type were comparable ($P > 0.05$, *t*-test). These findings indicate that, in *Xoo*, *rsmA*$_{Xoo}$ is involved in the production of extracellular endoglucanase and amylase, but not of xylanase and protease.

rsmA$_{Xoo}$ represses bacterial aggregation and cell adhesion

To determine whether RsmA$_{Xoo}$ is involved in biofilm formation, we observed the formation of cell aggregates in a liquid medium within a glass bottle and measured the quantity of cells that adhered to the glass surface indirectly, that is by staining. The wild-type strain 13751 and the complemented strain GXC2790 did not form aggregates in Luria–Bertani medium (Dow *et al*., 2003), whereas the mutant strain GXN2790 did, and formed readily visible clumps in the liquid medium (Fig. 4a), suggesting that it can form a biofilm. The extent of cell adhesion on the glass surface was measured by staining with crystal violet (Jackson *et al*., 2002), and was found to be significantly greater in *rsmA*$_{Xoo}$ mutant cells than in the wild-type and the complemented strain ($P < 0.01$, *t*-test) (Fig. 4b). These data suggest that *rsmA*$_{Xoo}$ may suppress *Xoo* biofilm formation.

rsmA$_{Xoo}$ positively regulates the expression of genes associated with pathogenicity and extracellular enzymes

To determine the molecular basis of biological changes in the phenotype caused by mutations in *rsmA*$_{Xoo}$, we measured the mRNA level of selected genes associated with pathogenicity and extracellular enzymes in the wild-type, *rsmA*$_{Xoo}$ mutant GXN2790 and the complemented strain GXC2790 by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). Unfortunately, we could not design primers to specifically test the expression of each gene encoding individual members of the AvrBs3/PthA family by RT-PCR. However, we did measure the total transcripts of the AvrBs3/PthA family in *Xoo* strains, and found them to be down-regulated in the *rsmA*$_{Xoo}$ mutant when compared with the wild-type strain (Figs 5 and S2, see Supporting Information). In contrast, the total transcripts of the AvrBs3/PthA family in the complemented strain GXC2790 were similar to those in the wild-type strain (Figs 5 and S2).

The levels of expression of *hrpB1*, *hrcU* and *hrcQ* (the first genes of three *hrp* operons in the *hrp* gene cluster), and of *hpa1*, *hpa2*, *hrpA*, *hrpF* and *hpaF*, in the complemented strain

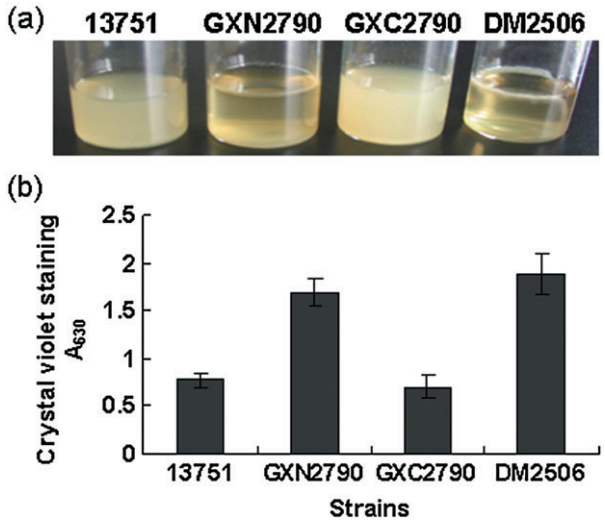

Fig. 4 *rsmA*$_{Xoo}$ represses cell aggregation and cell adhesion. (a) In the cell aggregate assay, the *rsmA*$_{Xoo}$ mutant GXN2790 and the *rsmA*$_{Xcc}$ mutant DM2506 (positive control) grew as aggregates on the surface of Luria–Bertani medium, whereas the wild-type strain 13751 and the complemented strain GXC2790 grew as dispersed cells. The results presented are from a representative experiment; similar results were obtained in the other two independent experiments. (b) Cells adhering to the glass surface. DM2506, the *rsmA*$_{Xcc}$ mutant of *Xanthomonas campestris* pv. *campestris* (*Xcc*), was included as the control. Three replicates were maintained for each experiment and each experiment was repeated three times. The data shown are the means ± standard deviation of the replicates.

GXC2790 were similar to those in the wild-type strain, but were down-regulated in the *rsmA*$_{Xoo}$ mutant GXN2790 (Figs 5 and S2). This difference indicates that *rsmA*$_{Xoo}$ positively regulates the expression of most *hrp* genes. However, the levels of expression of *hrpE1*, the first gene of the *hrpE* operon (data not shown), and of *hrpG* and *hrpX*, were about the same in all three strains (Figs 5 and S2). The expression of the *xrvA* gene was down-regulated in the *rsmA*$_{Xoo}$ mutant, but did not differ between the wild-type and the complemented strain. Yet, the expression level of *rpfB*, which is involved in DSF synthesis, was found to be down-regulated in the *rsmA*$_{Xoo}$ mutant GXN2790 (Figs 5 and S2).

The expression levels of the cellulase gene *eglXo*B, the glycogen biosynthesis gene *glgA* and *XOO0175*, the homologue of the *Xac* α-amylase gene *XAC0154*, were down-regulated in the *rsmA*$_{Xoo}$ mutant. However, the expression of these genes did not differ between the wild-type and the complemented strain. Furthermore, we found no differences in the levels of expression of *gumB*, *rpfC*, *rpfF* and *rpfG* in GXN2790 compared with the wild-type and the complemented strain (Figs 5 and S2).

DISCUSSION

In this study, inactivation of the *rsmA*$_{Xoo}$ gene in *Xoo* resulted in an almost complete loss of virulence on rice and in the failure to elicit an HR in a nonhost, namely castor-oil plant, indicating that *rsmA*$_{Xoo}$ is essential for the virulence of *Xoo* and for its ability to elicit an HR. The altered virulence can be partly explained by the reduced expression of genes associated with pathogenicity and the production of virulence factors, such as extracellular enzymes, observed in the *rsmA*$_{Xoo}$ mutant. Although the phenotypes and expression of some selected genes observed in the *rsmA*$_{Xoo}$ mutant were similar to those observed for *Xcc* (Chao *et al*., 2008), the *rsmA*$_{Xoo}$ gene may regulate the virulence of *Xoo* through a mechanism or pathway different from that in *rsmA*$_{Xcc}$ in *Xcc*, because members of the AvrBs3/PthA transcriptional activation-like (TAL) effector family in *Xoo* play an important role in the pathogenicity of the organism to rice, whereas their presence in *Xcc* has not been confirmed (Büttner and Bonas, 2010; White and Yang, 2009; White *et al*., 2009). In the present study, we found that the total transcripts of the AvrBs3/PthA family in the *rsmA*$_{Xoo}$ mutant were less than those in the wild-type strain, indicating that the expression of certain gene(s) encoding members of the AvrBs3/PthA family was reduced in the mutant, although we could not identify the affected gene(s). A proteomic approach is needed to determine the effect of mutations in *rsmA*$_{Xoo}$ on the expression of proteins, particularly those of the AvrBs3/PthA family, in *Xoo*.

What is particularly noteworthy is that *rsmA*$_{Xoo}$ positively regulates DSF production in *Xoo*. It has been reported previously that DSF promotes EPS production and xylanase activity in *Xoo* (He *et al*., 2010). Although we did not observe such altered production of extracellular xylanase by the *rsmA*$_{Xoo}$ mutant in our experiments, we did observe that EPS production in the *rsmA*$_{Xoo}$ mutant of *Xoo* was reduced, whereas the expression of genes such as *gumB*, *rpfC*, *rpfG* and *rpfF*, which are known to be involved in EPS production, was unaffected. Although the decreased production of EPS may be the result of reduced production of DSF, the precise mechanism by which *rsmA*$_{Xoo}$ regulates EPS production remains unknown. Furthermore, the expression of *rpfB*, which is involved in DSF synthesis (Barber *et al*., 1997), was down-regulated in the *rsmA*$_{Xoo}$ mutant, whereas the expression of *rpfF*, a key gene required for DSF biosynthesis (Barber *et al*., 1997; He *et al*., 2010), was unaffected. The *rpfB* and *rpfF* genes are co-transcribed from a promoter upstream of *rpfB*, but *rpfF* also has its own promoter (Slater *et al*., 2000). The transcriptional level of *rpfF* in the *rsmA*$_{Xoo}$ mutant may come partly from the transcription of *rpfF* from its own promoter, which may not be regulated by *rsmA*$_{Xoo}$. It is also possible that the expression of *rpfF* is regulated post-transcriptionally by RsmA$_{Xoo}$.

The expression of *xrvA*, which encodes a virulence regulator (Feng *et al*., 2009), the cellulase gene *eglXoB* and *XOO0175*, which is a homologue of the *Xac* α-amylase gene *XAC0154*, was down-regulated in the *rsmA*$_{Xoo}$ mutant. The *xrvA* mutant of *Xoo* showed significantly reduced virulence to the host and

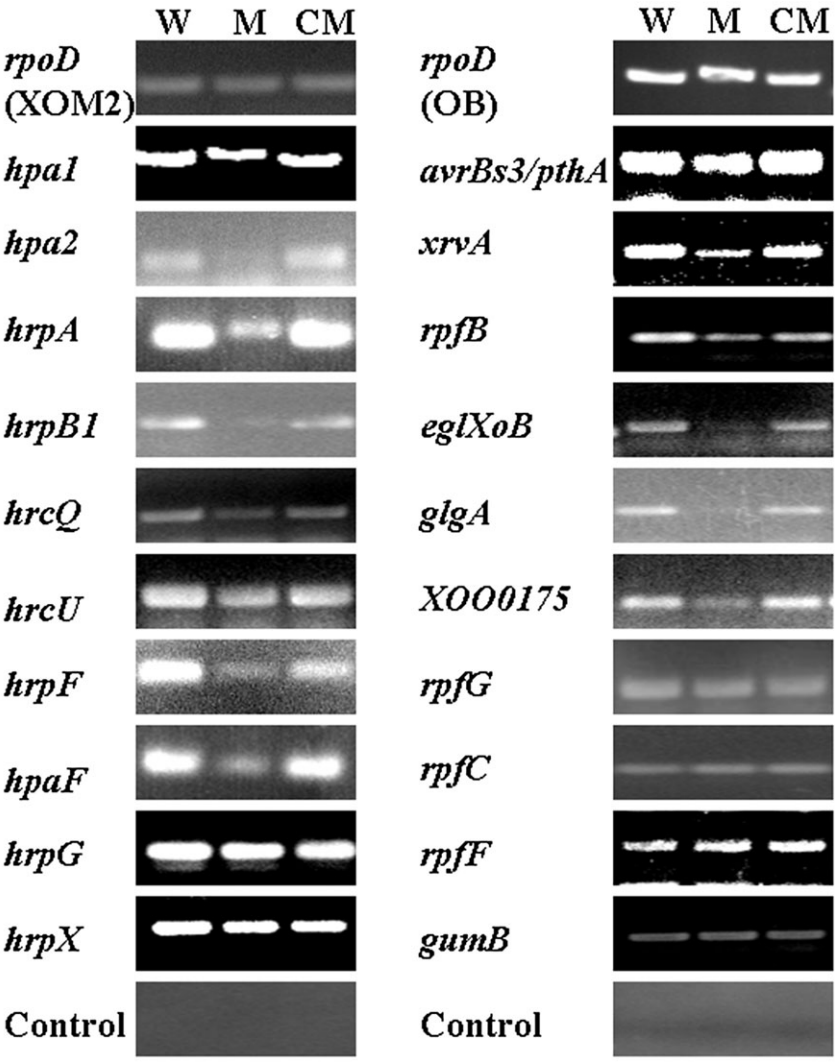

Fig. 5 Expression level of genes associated with virulence, type III secretion system (T3SS), extracellular enzymes and diffusible signal factor (DSF) in *Xanthomonas oryzae pv. oryzae* strains. The expression of *rpoD*, a housekeeping gene encoding the σ factor of RNA polymerase, in each strain was included as the control. The experiment was repeated four times. The results presented are from one experiment; similar results were obtained in all other independent experiments. The results of the other two independent experiments are presented in Fig. S2. Control means a negative control (without addition of reverse transcriptase). XOM2 and OB represent XOM2 and OB media for growing the bacteria for RNA extraction, respectively. WT, wild-type strain 13751; M, *rsmA*$_{Xoo}$ mutant strain GXN2790; CM, complemented strain GXC2790.

delayed induction of HR in a nonhost (Feng *et al*., 2009). The *eglXoB* gene is involved in the virulence of *Xoo* (Hu *et al*., 2007), but the role of *XOO0175* is unclear. Screening the transposon-based mutant library of *X. citri* ssp. *citri* revealed that a mutation of *XAC0798*, which encodes an α-amylase, affected virulence and induced an enhanced hypersensitive-like response (Laia *et al*., 2009).

The expression of *glgA*, a homologue of which is involved in glycogen biosynthesis in *E. coli* (Yang *et al*., 1996), was reduced in the *rsmA*$_{Xoo}$ mutant despite the greater accumulation of glycogen. The expression of *glgB*, which encodes a 1,4-α-glucan branching enzyme, is reduced in the *rsmA*$_{Xcc}$ mutant of *Xcc* (Chao *et al*., 2008). In *E. coli*, the *glgCAP* operon is involved in glycogen biosynthesis, and CsrA has been shown to directly suppress the expression of this operon (Baker *et al*., 2002). However, our sequence analysis did not identify any homologue of *glgC* or *glgP* in the sequenced genome of *Xoo* strains. Therefore, the mechanism by which *rsmA*$_{Xoo}$ affects the expression of *glgA* and glycogen accumulation in *Xoo* remains unclear.

In bacteria, the RsmA/CsrA proteins regulate translation by binding specifically to conserved sequences at or near the Shine–Dalgarno sequence of target mRNAs, thus affecting mRNA translation and/or stability. The effect of RsmA/CsrA is relieved by small RNAs (sRNAs) that sequester and antagonize these proteins (Lapouge *et al*., 2008). To better understand the function and regulatory network of RsmA$_{Xoo}$ in *Xoo*, it is important to identify the RsmA$_{Xoo}$-binding sRNAs. Surprisingly, we found no sequence homologous to the sRNA-encoding sequences in the sequenced genome of *Xoo* strains. Nevertheless, the detailed mechanism of RsmA$_{Xoo}$ action in *Xoo* merits further studies.

EXPERIMENTAL PROCEDURES

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. The strains of *E. coli* were cultivated in Luria–Bertani medium (Miller, 1972) at 37°C. *Xoo* strains were grown in OB medium (Tang *et al*., 1996) or XOM2 medium (Tsuge *et al*., 2002) at 28°C. The *Xcc rpfF* mutant strain 8523 was inoculated into peptone–yeast extract–glycerol broth (NYGB) (Daniels *et al*., 1984). Antibiotics were used as described previously (Feng *et al*., 2009).

DNA manipulation

DNA manipulation was performed following the procedures described by Sambrook and Russell (2001). Conjugation between the *Xoo* and *E. coli* strains was carried out as described by Turner *et al*. (1985). Restriction enzymes and DNA ligase were used in accordance with the manufacturer's instructions (Promega, Shanghai, China).

Mutant construction

The nonpolar mutant of $rsmA_{Xoo}$ in the *Xoo* strain 13751 (Tang *et al*., 1996) was generated by homologous integration with a suicide plasmid using pKMob18GII as a vector (Katzen *et al*., 1999), as described previously (Windgassen *et al*., 2000). A 131-bp internal fragment of the $rsmA_{Xoo}$ gene (from 7 bp downstream of the start codon to 137 bp) was amplified using total DNA of strain 13751 as the template and the primer set $rsmA_{Xoo}$IF/$rsmA_{Xoo}$IR (Table S1, see Supporting Information). The amplified DNA fragment was ligated to the plasmid vector pKMob18GII to create the recombinant plasmid pK$rsmA_{Xoo}$ (Table 1), and confirmed by sequencing. The plasmid pK$rsmA_{Xoo}$ was transferred into strain 13751 by triparental conjugation using pRK2073 (Leong *et al*., 1982) as the helper plasmid. With a mutation in the $rsmA_{Xoo}$ gene, transconjugants were confirmed by PCR using total DNA as the template and the primer pair pKMob18GIIF/$rsmA_{Xoo}$YR (Table S1). Total DNA from the wild-type strain as a template was used as a negative control. The expected 393-bp PCR product was further confirmed by sequencing. One of the confirmed mutants, designated as GXN2790 (Table 1), was used for further study.

Complementation of the $rsmA_{Xoo}$ mutant

A 703-bp DNA fragment containing the entire $rsmA_{Xoo}$ gene (from 447 bp upstream of the start codon to 43 bp downstream of the stop codon) was amplified by PCR using total DNA from the wild-type strain 13751 as the template and the primer set $rsmA_{Xoo}$CF/$rsmA_{Xoo}$CR (Table S1). The PCR product was then sequenced. The sequence of the 703-bp DNA fragment was identical to the corresponding DNA sequence containing $rsmA_{Xoo}$ in the sequenced genomes of *Xoo* strains MAFF311018, KACC10331 and PXO99^{A} (accession numbers AP008229, AE013598 and CP000967, respectively). The amplified DNA fragment was then cloned into pLAFR6 to generate the recombinant plasmid, named pL6rsmA_{Xoo} (Table 1), which was then transferred into the mutant GXN2790 by triparental conjugation (Turner *et al*., 1985). The confirmed transconjugant was designated as GXC2790 (Table 1) and chosen for further study.

RT-PCR

Total RNA was extracted from wild-type *Xoo* 13751, mutant GXN2790 and the complemented strain GXC2790, grown either in liquid medium OB or in *hrp*-inducing medium XOM2, as described by Feng *et al*. (2009). An equal amount of RNA from each strain was converted to cDNA by RT-PCR (Takara Biotechnology Co. Ltd., Dalian, China). The cDNAs were used as templates for amplification by PCR for 30 cycles with Ex-Taq polymerase (Takara Biotechnology Co. Ltd.). The primers used are listed in Table S1. PCRs using total DNA as template and total RNA without reverse transcriptase were used as positive and negative controls, respectively. The internal fragment of the 16S rRNA gene or *rpoD*, a housekeeping gene encoding the σ factor of RNA polymerase, was amplified simultaneously as an expression level control. The resulting amplified products were analysed on 1.2% agarose gel.

Plant assays

The virulence of *Xoo* strains was tested on hybrid rice cultivar Teyou 63, grown in a glasshouse with 12 h of light alternating with 12 h of darkness at temperatures of 28°C during the day and 25°C at night, using the leaf-clipping method (Kauffman *et al*., 1973). Bacterial cells were grown in OB medium at 28°C with shaking at 200 r.p.m. for 20 h (until the exponential growth phase was reached). The cell concentration was adjusted to an optical density of 0.001 (1×10^6 cfu/mL) or 0.1 (1×10^8 cfu/mL) at 600 nm. Thirty leaves were inoculated with each *Xoo* strain in each treatment and maintained at 100% humidity for 24 h following the inoculation, after which the plants were maintained under the growth conditions described above. The lesion length was measured 14 days after inoculation. Each treatment was repeated three times in each experiment. The same experiment was repeated three times.

HR was tested on a nonhost, namely castor-oil plant (*R. communis*), as described previously (Feng *et al*., 2009). The plants were inoculated by infiltrating approximately 20 μL of the bacterial suspension (5×10^7 cfu/mL or 5×10^8 cfu/mL) in 10 mM sodium phosphate buffer into the leaves using a 1-mL blunt-end plastic syringe. The inoculated plants were maintained in a glasshouse with a 12 h day–night cycle of illumination using a fluo-

rescent lamp at a constant temperature of 28°C. Plants were observed at 12, 24, 36 and 48 h after inoculation. The HR symptoms were photographed 48 h after inoculation. At least four plants were inoculated in each experiment, and each experiment was repeated three times.

Growth of bacteria in media and *in planta*

The growth of *Xoo* strains was measured in OB and XOM2 media. Bacterial cells were first grown in OB medium at 28°C with shaking at 200 r.p.m. for 20 h, that is until the exponential growth phase was reached. The cell concentration was adjusted to an optical density of 1.0 (1×10^9 cfu/mL) at 600 nm. The cell suspension (2 mL) was transferred to 100 mL of OB or XOM2 medium containing appropriate antibiotics. Three replications were maintained for each strain. The optical density was measured at 600 nm at 2- and 4-h intervals for cultures in OB and XOM2 media, respectively.

Bacterial growth was measured in the leaf tissue of Teyou 63 inoculated with the *Xoo* strains (1×10^8 cfu/mL; $OD_{600} = 0.1$) by the leaf-clipping method (Kauffman *et al*., 1973). The growth was measured at 2-day intervals up to 16 days after inoculation. Leaf samples (three leaves to a sample) were homogenized in 5 mL of sterile water. The diluted homogenate was plated onto OB agar supplemented with streptomycin (for the wild-type), streptomycin plus kanamycin (for the mutant) or streptomycin plus kanamycin and tetracycline (for the complemented strain). The number of colonies on each plate was counted after incubation at 28°C for 3 days. The bacterial cfu was calculated for each rice leaf. The procedure was repeated three times for each sample. The data shown are the average of three replications with standard deviation.

Assessment of extracellular enzyme activity, EPS production, DSF biosynthesis and glycogen accumulation

The extracellular activity of endoglucanase, xylanase and amylase in *Xoo* strains was measured essentially as described by Feng *et al*. (2009). Briefly, *Xoo* strains were cultured in OB medium for 24 h for the measurement of endoglucanase and amylase activity, and for 48 h for the measurement of xylanase activity. The culture supernatant (100 μL) was added to 400 μL of the appropriate buffer containing 1% (w/v) carboxymethylcellulose (CMC, Sigma, St. Louis, MO, USA) for endoglucanase, 1% (w/v) birchwood xylan (Sigma) for xylanase or 1% (w/v) soluble starch (Sinopharm Chemical Reagent Co. Ltd., Shangshai, China) for amylase. The reactions were carried out for 30 min at 28°C. The released reducing sugars were measured as D-glucose or D-xylose equivalents, as described by Miller (1959). One unit (U) of endoglucanase/xylanase/amylase activity was defined as the amount of enzyme releasing 1 μmol of reducing sugar per minute.

To measure EPS production, *Xoo* strains were cultured in conical flasks containing 100 mL of OB medium supplemented with 2% (w/v) glucose instead of sucrose at 28°C for 4 days, with shaking at 200 r.p.m. EPS was precipitated from the culture supernatant by the addition of three volumes of 95% ethanol. The pelleted EPS was washed with 70% ethanol, dried at 60°C and weighed. Three flasks were inoculated for each strain in each experiment, and each experiment was repeated three times.

DSF was extracted into ethyl acetate (Barber *et al*., 1997) from culture supernatants of *Xoo* strains grown in OB medium for 48 h. The ethyl acetate extracts were evaporated to dryness and the samples were resuspended in methanol. The *Xcc rpfF* mutant strain 8523 (Table 1) was inoculated into NYGB supplemented with the DSF extracts, and cultured for 24 h. The restored extracellular endoglucanase activity produced by mutant strain 8523 was measured as described above for endoglucanase activity.

The intracellular accumulation of glycogen was initially determined by the method described by Chao *et al*. (2008), and then measured as described by Feng *et al*. (2009).

Cell aggregation and adhesion assay

Bacterial cell aggregates that formed on the surface of the medium or adhered to the glass surface were surveyed using the method described by Rhim (1983). The extent of adhesion was measured using the methods described by Jackson *et al*. (2002) and Chao *et al*. (2008).

Nucleotide sequence accession number

The *rsmA*$_{Xoo}$ sequence of *Xoo* strain 13751 was deposited into the GenBank database (accession number HM988729).

ACKNOWLEDGEMENTS

This work was supported by the 973 Program of the Ministry of Science and Technology of China (2006CB101902).

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SUPPORTING INFORMATION

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

Fig. S1 Genetic organization of *rsmA/csrA* loci in the genomes of *Xanthomonas oryzae* pv. *oryzae*, *X. campestris* pv. *campestris*, *Escherichia coli*, *X. axonopodis* pv. *citri* and *X. campestris* pv. *vesicatoria*. The DNA sequence containing genes *alaS*, $rsmA_{Xoo}$ and *tRNA-Ser* in *X. oryzae* pv. *oryzae* (*Xoo*) strain 13751 was obtained by sequencing the polymerase chain reaction (PCR) products amplified using total DNA from strain 13751 as the template and the primers (data not shown) designed on the basis of the sequence from the genome of *Xoo* strain MAFF311018 (accession number AP008229). This DNA sequence was identical to the corresponding sequence in the genome of *Xoo* strain MAFF311018. The DNA sequence containing *rsmA/csrA* loci in the other bacteria is from the sequenced genomes of *X. campestris* pv. *campestris* strain 8004 (accession number CP000050), *E. coli* strain K12 substrain MG1655 (accession number U00096), *X. axonopodis* pv. *citri* strain 306 (accession number AE008923) and *X. campestris* pv. *vesicatoria* strain 85-10 (accession number AM039952). The genes *alaS* and *tRNA-Ser* encode alanyl-tRNA synthetase and tRNA-Ser, respectively. Horizontal black bars indicate the sequences used in plasmid construction.

Fig. S2 Expression level of genes associated with virulence, the type III secretion system (T3SS), extracellular enzymes and diffusible signal factor (DSF) in *Xanthomonas oryzae* pv. *oryzae* strains detected in independent experiments. (a) One independent experiment carried out by one individual. The expression of 16S rRNA in each strain was included as the control. The negative control contained no reverse transcriptase, and no bands appeared (results not shown). XOM2 and OB represent XOM2 medium and OB medium for growing the bacteria for RNA extraction, respectively. WT, wild-type strain 13751; M, $rsmA_{Xoo}$ mutant strain GXN2790; CM, complemented strain GXC2790. (b) Another independent experiment carried out by another individual. The expression of *rpoD*, a housekeeping gene encoding the σ factor of RNA polymerase, in each strain was included as the control. Control means a negative control (without the addition of reverse transcriptase). XOM2 and OB represent XOM2 medium and OB medium for growing the bacteria for RNA extraction, respectively. WT, wild-type strain 13751; M, $rsmA_{Xoo}$ mutant strain GXN2790; CM, complemented strain GXC2790.

Table S1 Primers used in this study.

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