The *rsmA*-like Gene *rsmA_{Xcc}* of *Xanthomonas campestris* pv. *campestris* Is Involved in the Control of Various Cellular Processes, Including Pathogenesis

Nai-Xia Chao,¹ Ke Wei,^{1,2} Qi Chen,¹ Qing-Lin Meng,¹ Dong-Jie Tang,¹ Yong-Qiang He,¹ Guang-Tao Lu,¹ Bo-Le Jiang,¹ Xiao-Xia Liang,¹ Jia-Xun Feng,¹ Baoshan Chen,¹ and Ji-Liang Tang¹

¹Guangxi Key Laboratory of Subtropical Bioresources Conservation and Utilization, The Key Laboratory of Ministry of Education for Microbial and Plant Genetic Engineering, and College of Life Science and Technology, and ²College of Agriculture, Guangxi University, 100 Daxue Road, Nanning Guangxi 530004, China

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RsmA is an RNA-binding protein functioning as a global post-transcriptional regulator of various cellular processes in bacteria and has been demonstrated to be an important virulence regulator in many animal bacterial pathogens. However, its function in other phytopathogenic bacteria is unclear, except for the *Erwinia carotovora* RsmA, which acts as a negative virulence regulator. In this work, we investigated the function of the *rsmA*-like gene, named *rsmA_{Xcc}*, of the phytopathogen *Xanthomonas campestris* pv. *campestris*. Deletion of *rsmA_{Xcc}* resulted in complete loss of virulence on the host plant Chinese radish, hypersensitive response on the nonhost plant pepper ECW-10R, and motility on the surface of an agar plate. The *rsmA_{Xcc}* mutant displayed a significant reduction in the production of extracellular amylase, endoglucanase, and polysaccharide, but a significant increase in intracellular glycogen accumulation and an enhanced bacterial aggregation and cell adhesion. Microarray hybridization and semiquantitative reverse-transcription polymerase chain reaction analysis showed that deletion of *rsmA_{Xcc}* led to significantly reduced expression of genes encoding the type III secretion system (T3SS), T3SS-effectors, and the bacterial aggregate dispersing enzyme endo- β -1,4-mannanase. These results suggest that *rsmA_{Xcc}* is involved in the control of various cellular processes, including pathogenesis of *X. campestris* pv. *campestris*.

The RNA-binding protein RsmA (repressor of secondary metabolism) in bacteria is a global post-transcriptional regulator playing an important role in the regulation of various cellular processes, most if not all associated with secondary metabolic systems. This regulator first was identified as a repressor of carbon metabolism in *Escherichia coli* and designated as CsrA (carbon storage regulator) (Romeo 1998; Romeo et al. 1993). Subsequently, the CsrA of *E. coli* was found to act as a post-transcriptional regulator that represses glycogen biosynthesis, gluconeogenesis, and peptide transport as well as

biofilm formation, and activates glycolysis, acetate metabolism, and cell motility (Dubey et al. 2003; Sabnis et al. 1995; Wei et al. 2001). In addition, RsmA/CsrA also has been demonstrated to be involved in the pathogenesis of many animal pathogens, including *Salmonella enterica* (Altier et al. 2000a), *Pseudomonas aeruginosa* (Pessi et al. 2001), *Legionella pneumophila* (Molofsky and Swanson 2003), *Proteus mirabilis* (Liaw et al. 2003), *Helicobacter pylori* (Barnard et al. 2004), *Listeria monocytogenes* (Milenbachs et al. 2004) and *Vibrio cholerae* (Lenz et al. 2005), and a plant pathogen, *Erwinia carotovora* (Chatterjee et al. 1995). The RsmA/CsrA of these pathogens negatively or positively regulates the expression of pathogenicity genes and the production of virulence determinants. For instance, the CsrA of *S. enterica* serovar Typhimurium positively regulates the expression of invasion genes and components of the virulence-associated type III secretion system (T3SS) (Altier et al. 2000a and b; Lawhon et al. 2003). Similarly, the RsmA of *Pseudomonas aeruginosa* positively regulates the T3SS (Mulcahy et al. 2006; O'Grady et al. 2006). In contrast, in the plant pathogen *E. carotovora*, RsmA represses the production of virulence factors such as pectate lyase, cellulase, and protease (Chatterjee et al. 1995) and the expression of some T3SS-related genes (Chatterjee et al. 2002).

Although RsmA/CsrA has been demonstrated to be a common virulence regulator for many animal bacterial pathogens, and whole-genome sequencing revealed that *rsmA/csrA* homologues are widely distributed in plant bacterial pathogens (da Silva et al. 2002; Feil et al. 2005; Lee et al. 2005; Qian et al. 2005; Simpson et al. 2000; Thieme et al. 2005), little is known about its role in the pathogenesis of phytopathogenic bacteria apart from *E. carotovora*. *Xanthomonas campestris* pv. *campestris* is the causal agent of black rot, one of the most severe diseases of cruciferous crops worldwide (Alvarez 2000). It has been used as a model bacterium for studying pathogen–host interactions for over two decades and some dozens of pathogenicity genes have been identified (Arlat et al. 1991; Daniels et al. 1984a and b; Dow and Daniels 1994; Dow et al. 2003; Qian et al. 2005; Tang et al. 2005; Tang et al. 1991; Wei et al. 2007). In this report, we present genetic evidence to demonstrate that the *rsmA/csrA* homologue in *X. campestris* pv. *campestris*, named *rsmA_{Xcc}*, acts as a global regulator that controls carbon metabolism, cell motility, bacterial aggregation, cell adhesion, and pathogenesis.

N.-X. Chao and K. Wei contributed equally to this work.

Corresponding author: J.-L. Tang; E-mail: jltang@gxu.edu.cn; Telephone: +86-771-3239566; Fax: +86-771-3239413.

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RESULTS

rsmA_{Xcc} is essential for the virulence and hypersensitive response of *X. campestris* pv. *campestris*.

In the genome of the *X. campestris* pv. *campestris* strain 8004, the open reading frame (ORF) XC_2506 (GenBank accession number AAY49556) was annotated as *rsmA*/*csrA* (hereafter *rsmA_{Xcc}*) (Qian et al. 2005). The deduced protein RsmA_{Xcc} displays 69, 69, and 67% identity to the *Escherichia coli* CsrA, *Erwinia carotovora* RsmA, and *P. aeruginosa* RsmA, respectively (Fig. 1). Protein secondary structure prediction (The Advanced Protein Secondary Structure Prediction Server) revealed that RsmA_{Xcc} shares almost identical elements with the RsmA/CsrA of *E. carotovora*, *Escherichia coli*, and *P. aeruginosa* defined by Gutiérrez and associates (2005) (Fig. 1). The *rsmA_{Xcc}* is located in anticlockwise orientation of the genome sequence between position 3035290 and position 3035502 of the *X. campestris* pv. *campestris* strain 8004 (Qian et al. 2005). The nearest genes upstream and downstream of *rsmA_{Xcc}* are *alaS* (encoding a putative alanyl-tRNA synthetase) and *mcpA* (encoding a putative chemotaxis protein), respectively. The genes *alaS*, *rsmA_{Xcc}*, and *mcpA* are in the same orientation and the spacers between *alaS* and *rsmA_{Xcc}*, and *rsmA_{Xcc}* and *mcpA* are 143 and 1,216 bp, respectively (Qian et al. 2005). Reverse-transcription polymerase chain reaction (RT-PCR) results revealed that these three genes are not co-transcribed (data not shown). Sequence comparison search showed that no other genes in the genome of the *X. campestris* pv. *campestris* strain 8004 display homologous DNA sequences with *rsmA*/*csrA*.

In order to study the biological function of *rsmA_{Xcc}*, an *rsmA_{Xcc}* deletion mutant was constructed from the *X. campestris* pv. *campestris* wild-type strain 8004 by marker exchange-eviction mutagenesis (Ried and Collmer 1987) and named DM2506 (Table 1). Simultaneously, a complemented strain,

designated as CDM2506, was constructed by transferring the recombinant plasmid pLrsmA_{Xcc}, which carries an entire wild-type *rsmA_{Xcc}* gene, into the mutant DM2506 (Table 1) (details below). To determine whether mutation in *rsmA_{Xcc}* has any effect on the growth of *X. campestris* pv. *campestris*, we examined the growth of the *rsmA_{Xcc}* mutant strain DM2506 and the wild-type strain 8004 in the minimal medium MMX and the rich medium NYG. The results showed that the *rsmA_{Xcc}* mutant and the wild-type strain grew identically in both media (Fig. 2A and B), indicating that *rsmA_{Xcc}* is not required for the growth of *X. campestris* pv. *campestris* in standard media. We also tested the competitive growth of the *rsmA_{Xcc}* mutant versus the wild-type strain in the minimal medium MMX. The result showed that the growth competitiveness of the *rsmA_{Xcc}* mutant is almost the same as that of the wild-type strain in the testing conditions (Fig. 2C).

The virulence of the *rsmA_{Xcc}* mutant DM2506 was determined on the host plant Chinese radish (*Raphanus sativus* L. var. radiculatus Pers.) by the leaf-clipping method (Dow et al. 2003). Ten days postinoculation, no disease symptoms were observed on the leaves inoculated with the mutant strain DM2506, whereas typical V-shaped disease symptoms with mean lesion lengths of 14.2 and 13.9 mm were observed on the leaves inoculated with the wild-type strain 8004 and the complemented strain CDM2506, respectively (Fig. 3A and B). The result of *t* tests showed that the mean lesion lengths caused by the complemented strain and the wild-type strain were not significantly different (*P* = 0.01). The virulence of these strains also was determined on another *X. campestris* pv. *campestris* host plant cabbage (*Brassica oleracea* L. var. JingFengNo1) by the same method, and similar results were observed (data not shown). These findings demonstrate that *rsmA_{Xcc}* is essential for *X. campestris* pv. *campestris* to cause disease on host plants. To evaluate the role of *rsmA_{Xcc}* in growth of *X. campestris* pv.

Fig. 1. Amino acid sequence comparison and secondary structure prediction of RsmA homologs from *Xanthomonas campestris* pv. *campestris* (RsmA-Xcc) (GenBank accession number AAY49556), *Pseudomonas aeruginosa* (RsmA-P. *aeruginosa*) (GenBank accession number NP_249596), *Escherichia coli* (CsrA-E. *coli*) (GenBank accession number NP_417176), and *Erwinia carotovora* (RsmA-E. *carotovora*) (GenBank accession number YP_051455). Black boxes denote identical amino acid residues. The secondary structure elements shown on top are based on the prediction results using the Advanced Protein Secondary Structure Prediction Server and the data published by Gutiérrez and associates (2005).

Table 1. Bacterial strains and plasmids used in this work

Strains or plasmids	Relevant characteristics ^z	Reference or source
<i>Escherichia coli</i>		
JM109	<i>RecA1</i> , <i>endA1</i> , <i>gyrA96</i> , <i>thi</i> , <i>supE44</i> , <i>relA1</i> Δ(<i>lac-proAB</i>)/F' [<i>traD36</i> , <i>lacI</i> ^q , <i>lacZ</i> ΔM15]	Yanisch-Perron et al. 1985
<i>Xanthomonas campestris</i> pv. <i>campestris</i>		
8004	Wild type, Rif ^r	Daniels et al. 1984
DM2506	As 8004, but the coding region of <i>rsmA_{Xcc}</i> (XC2506) has been deleted, Rif ^r	This study
CDM2506	DM2506 harboring pLrsmA _{Xcc} , Rif ^r , Tc ^r	This study
<i>hrcV</i> ⁻	As 8004, but the coding region of <i>hrcV</i> (XC3013) has been deleted, Rif ^r	Laboratory collection
Plasmids		
pLAFR3	Broad host range cloning vector, Tc ^r	Huynh et al. 1989
pRK2073	Helper plasmid, Tra ⁺ , Mob ⁺ , ColE1, Spc ^r	Leong et al. 1982
pK18mob	Suicide plasmid in <i>Xanthomonas</i> , Kan ^r	Schafer et al. 1994
pKsacB	pK18mob containing a <i>sacB</i> gene, Kan ^r	Laboratory collection
pKrsmAFL	pKsacB containing 1.6-kb flanking sequence of <i>rsmA_{Xcc}</i> , Kan ^r	This study
pLrsmA _{Xcc}	pLAFR3 containing the <i>rsmA_{Xcc}</i> gene, Tc ^r	This study

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

campestris inside the host, the populations of the *rsmA_{Xcc}* mutant DM2506, the wild-type strain 8004, and the complemented strain CDM2506 in the inoculated radish leaves were compared. The results showed that the bacterial number of the mutant strain DM2506 recovered from the infected leaves was significantly fewer than that of the wild type at each of the test points ($P = 0.01$ by *t* test) (Fig. 3D). The *rsmA_{Xcc}* mutant hardly multiplied inside the host whereas the wild-type strain multiplied well (Fig. 3D). There was no significant difference between the growth rates of the complemented strain CDM2506 and the wild-type strain 8004 (Fig. 3D), indicating that the growth capacity of the mutant in planta could be restored by *rsmA_{Xcc}* in trans. These results demonstrate that *rsmA_{Xcc}* is essential for *X. campestris* pv. *campestris* to proliferate in host plants.

To investigate whether *rsmA_{Xcc}* is required for the hypersensitive response (HR) of the pathogen, the *rsmA_{Xcc}* mutant DM2506 was inoculated on the pepper cv. ECW-10R, a non-host plant typically used to test the HR of *X. campestris* pv. *campestris* (Castañeda et al. 2005; Newman et al. 2001). The result displayed that the *rsmA_{Xcc}* mutant DM2506 was unable to trigger an HR on the inoculated pepper leaves (Fig. 3C). However, the complemented strain CDM2506 could induce a wild-type HR (Fig. 3C), suggesting that the HR-induction capacity of the mutant could be restored completely by *rsmA_{Xcc}* in trans. These results reveal that *rsmA_{Xcc}* is required for *X. campestris* pv. *campestris* to induce HR on the nonhost plant pepper ECW-10R.

***rsmA_{Xcc}* is involved in the production of extracellular amylase, endoglucanase, and polysaccharide.**

Extracellular polysaccharide (EPS) is an important virulence factor, and extracellular enzymes, including protease, endoglucanase, and amylase, collectively contribute to the virulence of *X. campestris* pv. *campestris* (Chan and Goodwin 1999; Dow and Daniels 1994). To verify whether the virulence-deficient *rsmA_{Xcc}* mutant exhibits any defects in production of these virulence factors, the EPS production and the extracellular enzymes' activity of the *rsmA_{Xcc}* mutant DM2506 were measured (discussed below). These results showed that the *rsmA_{Xcc}* mutant and the wild type displayed a similar extracellular protease activity, whereas the EPS production and activity of the extracellular endoglucanase and amylase of the *rsmA_{Xcc}* mutant were significantly diminished ($P = 0.01$ by *t* test) compared with those of the wild-type strain 8004 (Table 2). The complemented strain CDM2506 produced a wild-type level on the EPS production and activity of the extracellular amylase and endoglucanase (Table 2). These findings reveal that the *X. campestris* pv. *campestris* *rsmA_{Xcc}* is involved in the production of extracellular amylase and endoglucanase as well as EPS, but not exoprotease. It seems that mutation in *rsmA_{Xcc}* does not affect the production of lipopolysaccharide (LPS), a cell surface carbohydrate that has been shown to be involved in the interaction of *X. campestris* pv. *campestris* with its host plants (Silipo et al. 2005), because the LPS pattern of the *rsmA_{Xcc}* mutant strain DM2506 is identical to that of the wild-type strain 8004 (data not shown).

***rsmA_{Xcc}* activates cell motility and represses cell adhesion, bacterial aggregation, and glycogen accumulation.**

It has been demonstrated that, in a variety of bacteria, RsmA/CsrA functions as a regulator of diverse cellular processes, including cell motility (Heurlier et al. 2004; Liaw et al. 2003), biofilm formation (Burrowes et al. 2006; Jackson et al. 2002; Romeo et al. 1993), and glycogen biosynthesis (Romeo et al. 1993). To find out whether the *X. campestris* pv. *campestris* *rsmA_{Xcc}* has similar functions, we compared the cell

motility, biofilm formation, and glycogen accumulation of the *rsmA_{Xcc}* mutant with those of the wild-type strain. Motility of *X. campestris* pv. *campestris* strains was examined on the NYG plates containing 2% (wt/vol) glucose solidified with 0.3% (wt/vol) agar. The results clearly showed that the *rsmA_{Xcc}* mutant was nonmobile whereas the wild type and the complemented strain migrated away from the inoculation points on the agar surface (Fig. 4A). These results suggest that *rsmA_{Xcc}* may activate cell motility of *X. campestris* pv. *campestris*.

Intracellular glycogen accumulation was determined by the iodine vapor staining method (Liu et al. 1997). The *rsmA_{Xcc}* mutant strain DM2506 was dark brown due to excess glycogen accumulation, whereas the complemented strain CDM2506 stained yellowish brown, similar to the staining pattern of the wild-type strain 8004 (Fig. 4B). This indicates that *rsmA_{Xcc}* represses glycogen accumulation inside the *X. campestris* pv. *campestris* cells.

Fig. 2. Growth characteristics of the *rsmA_{Xcc}* mutant and the wild-type strain in different conditions. **A**, Rich medium NYG (Daniels et al. 1984a) and **B**, minimal medium MMX (Daniels et al. 1984b); Δ = the *rsmA_{Xcc}* mutant DM2506 and $\blacklozenge$ = wild-type strain 8004. **C**, Competitive growth between the *rsmA_{Xcc}* mutant and the wild-type strain in MMX medium.

The biofilm formation of the *X. campestris* pv. *campestris* strains was surveyed by observation of the cell aggregate formation in medium and quantitative analysis of cell adhesion on glass surface. It has been shown that the wild-type strain 8004 does not form aggregates in L medium (Dow et al. 2003). However, the *rsmA_{Xcc}* mutant strain DM2506 grew in an aggregated state and formed readily observable clumps, suggesting that the mutant formed biofilm (Fig. 4C). The complemented

strain CDM2506 grew similarly to the wild type, in a dispersed fashion (Fig. 4C). Cell adhesion on glass surfaces was analyzed quantitatively by the crystal violet staining method (Jackson et al. 2002). The results showed that the *rsmA_{Xcc}* mutant cells attached to the glass surface were more significant than those of the wild type and the complemented strain, although the three strains exhibited a similar growth rate in the testing conditions (Fig. 4D). These findings suggest that

Fig. 3. *rsmA_{Xcc}* is essential for *Xanthomonas campestris* pv. *campestris* to cause disease on host plant and hypersensitive response (HR) on nonhost plant. **A**, Black rot symptoms caused by *X. campestris* pv. *campestris* strains on inoculated leaves of the host plant Chinese radish. Photographs were taken on day 10 postinoculation. **B**, Average lesion lengths caused by *X. campestris* pv. *campestris* strains. Values are the mean $\pm$ standard deviation (SD) from three repeats, each with 60 leaves. **C**, HR induced on nonhost plant pepper leaves (*Capsicum annuum* cv. ECW-10R) by *X. campestris* pv. *campestris* strains. Approximately 5 μ l of bacterial culture suspended in 10 mM sodium phosphate buffer was infiltrated into the leaf mesophyll tissue with a blunt-end plastic syringe. The photograph was taken 48 h after infiltration. Three replications were done in each experiment, and each experiment was repeated three times. The results presented are from a representative experiment and similar results were obtained in all other independent experiments. The text under each row of pictures describes the cell concentrations used for inoculation and the observation time (hours postinoculation). The type III-deficient strain (*hrcV*⁻) was used as a negative control. **D**, Growth of bacteria in inoculated leaves. Bacteria were recovered from the inoculated leaves every day in a period of 9 days postinoculation. Data are the mean $\pm$ SD from three repeats; $\blacktriangle$ = wild-type strain 8004, $\blacksquare$ = the *rsmA_{Xcc}* mutant DM2506, and $\square$ = the complemented strain CDM2506.

Table 2. Quantification of extracellular polysaccharide and enzymes produced by *Xanthomonas campestris* pv. *campestris* strains^z

Strains	Extracellular protease (A ₃₆₆)	Extracellular endoglucanase (U)	Extracellular amylase (U)	Extracellular polysaccharide (g/liter)
8004 (wild type)	0.14 $\pm$ 0.02 A	0.46 $\pm$ 0.04 A	0.26 $\pm$ 0.03 A	12.3 $\pm$ 1.3 A
DM2506(<i>rsmA_{Xcc}</i> mutant)	0.18 $\pm$ 0.03 A	0.07 $\pm$ 0.01 B	0.05 $\pm$ 0.01 B	5.4 $\pm$ 1.9 B
CDM2506(complemented strain)	0.16 $\pm$ 0.04 A	0.54 $\pm$ 0.05 A	0.30 $\pm$ 0.07 A	11.5 $\pm$ 0.6 A

^z Data are the mean $\pm$ standard deviation of triplicate measurements; the different letters in each data column indicate significant differences at *P* = 0.01. The experiment was repeated twice and similar results were obtained.

rsmA_{Xcc} may repress the biofilm formation of *X. campestris* pv. *campestris*.

***rsmA_{Xcc}* is required for expression of the genes encoding T3SS, T3SS effectors, and the bacterial aggregate dispersing enzyme endo- β -1,4-mannanase.**

To facilitate the investigation of the molecular basis of the biological phenotype changes caused by the mutation in

rsmA_{Xcc}, we compared the gene expression profiles of the *rsmA_{Xcc}* mutant with the wild-type strain by a whole-genome microarray hybridization analysis. Because all of the identified bacterial RsmAs/CsrAs have been demonstrated to regulate gene expression at the translational level, our major concern in performing transcriptome analysis is to seek clues to the genes whose expression may be affected indirectly by *rsmA_{Xcc}*. For our interests, with particular respect to the pathogenicity of

Fig. 4. *rsmA_{Xcc}* activates cell motility and represses intracellular glycogen accumulation, cell aggregation, and cell adhesion. **A**, Cell motility on plates; the deletion mutant of *rsmA_{Xcc}* (DM2506) lost motility and the complemented strain CDM2506 (a plasmid expressing a wild-type RsmA_{Xcc} in the *rsmA_{Xcc}* mutant) showed wild-type motility. **B**, Intracellular glycogen accumulation measured by iodine vapor staining. Overnight culture (2 μ l) was spotted onto NYG plates containing 2% (wt/vol) glucose. Plates were stained with iodine vapor after incubation at 28°C for 4 days. **C**, Cell aggregate assay; the *rsmA_{Xcc}* mutant DM2506 grew in an aggregated fashion in the surface of L medium, while the wild-type strain 8004 and the complemented strain CDM2506 grew in a dispersed fashion. **D**, Cell adhesion assay on glass surface; 100 μ l of overnight culture of each *X. campestris* pv. *campestris* strain was inoculated into 5 ml of NYG liquid medium in a 25-ml universal tube. After being incubated steadily at 28°C for 24 h, the medium was discarded, the tube was rinsed three times with water, and the bound cells were stained with crystal violet. Finally, the dye was solubilized with 33% acetic acid, and absorbance at 630 nm was determined using a spectrophotometer. For all these experiments, three replicates were done in each experiment and each experiment was repeated three times. **A**, **B**, and **C**, Data presented were from a representative experiment and similar results were obtained in all other independent experiments. **D**, Values presented are the means $\pm$ standard deviations from three repeats.

X. campestris pv. *campestris*, we cultured bacterial cells in the minimal medium MMX, a condition where most of the known virulence genes are induced (Astua-Monge et al. 2000, 2005; Tang et al. 2006), and RNAs were isolated from the cells at the stationary phase. Microarray hybridization was carried out as described below, and the results showed that, of the 4,186 genes present on the array, 183 displayed a statistically significant ($P = 0.05$) increase and 257 displayed a significant ($P = 0.05$) decrease greater than twofold at mRNA level by *rsmA_{Xcc}* mutation (Supplemental Table S1). These expression-altered genes in the *rsmA_{Xcc}* mutant are distributed over all the functional categories of the *X. campestris* pv. *campestris* genes (Table 3) (Qian et al. 2005).

Consistent with the observation that the *rsmA_{Xcc}* mutant completely lost virulence and HR, the expression of many T3SS-related genes was significantly reduced in the mutant. These genes include 23 *hrp/hrc/hpa* genes and 4 avirulence genes: *avrBs1.1* (Xu et al. 2006), *avrXccC* (Castañeda et al., 2005), *avrAC_{Xcc8004}* (Xu et al. 2008), and *avrXccE1* (He et al. 2007) (Table 4). The decreased expression of these genes in *rsmA_{Xcc}* mutant was further confirmed by semiquantitative RT-PCR (Table 4). Interestingly, the microarray hybridization and RT-PCR results showed that, in the *rsmA_{Xcc}* mutant and the wild-type strain, the transcription of the two key *hrp* regulatory genes, *hrpG* and *hrpX*, was at the same level (Table 4), indicating that *rsmA_{Xcc}* might not regulate the transcription of *hrpG* and *hrpX*.

Similarly, consistent with the observation that the *rsmA_{Xcc}* mutant showed enhanced cell aggregation and loss of cell motility, the expression of the *manA* gene encoding the cell aggregate dispersing enzyme endo- β -1,4-mannanase (Dow et al. 2003) and several genes predicted to be involved in cell motility, such as *cheY* (XC2130) and *cheB* (XC1290, XC2163), was reduced (Table 4). Apart from *cheY*, *cheB*, and *hpaR* (Wei et al. 2007), there are 17 other putative regulatory genes whose expression was altered in the *rsmA_{Xcc}* mutant (Table 5). Among these genes, six encode proteins homologues to two-component regulatory systems. To clarify the function of these regulatory genes needs further investigation.

To our surprise, in contrast to more glycogen accumulation in the *rsmA_{Xcc}* mutant, the expression of *glgB* encoding 1,4- α -glucan branching enzyme, which has been demonstrated to be involved in glycogen biosynthesis in *E. coli*, was reduced in the mutant. The microarray results showed that none of the genes known to be involved in production of EPS and extracellular enzymes of *X. campestris* displayed altered expression

in the *rsmA_{Xcc}* mutant under the testing conditions. This suggests that *RsmA_{Xcc}* may affect the genes related to these cellular processes posttranscriptionally or by an unknown regulatory mechanism. Interestingly, the expression of a large number of genes encoding hypothetical proteins was altered in the *rsmA_{Xcc}* mutant (Table 3). The function of these genes needs to be clarified further.

DISCUSSION

The genome of the *X. campestris* pv. *campestris* strain 8004 possesses a unique *rsmA/csrA*-homologous gene (Qian et al. 2005). In this work, we have demonstrated genetically that this *rsmA/csrA* homologue is involved in various cellular processes, including carbon metabolism, cell motility, bacterial aggregate formation, cell adhesion, and pathogenesis of *X. campestris* pv. *campestris*. Inactivation of this gene resulted in an enhanced glycogen accumulation, cell adhesion, and bacterial aggregation, and a significant reduction in production of extracellular enzymes and EPS, as well as a significant loss of cell motility and T3SS function. On the basis of structural and phenotypic similarities between this gene and other identified bacterial *rsmA/csrA*, we considered it to be an *rsmA/csrA*-like gene and named it *rsmA_{Xcc}*, although no RNA-binding evidence for *RsmA_{Xcc}* has been obtained (see discussion below).

The *rsmA_{Xcc}* mutant completely lost virulence on host plants tested and HR on the nonhost plant pepper ECW-10R, indicating that *rsmA_{Xcc}* is essential for the pathogenicity and HR of *X. campestris* pv. *campestris*. The pathogenesis of *X. campestris* pv. *campestris* is a complex process involving several steps that mainly include invading host plants through wounds and natural openings, multiplying in the intercellular spaces, spreading via vascular system, and manifesting disease symptoms (Chan and Goodwin 1999). Since the 1980s, a large number of pathogenicity-related genes and virulence factors have been identified from *X. campestris* pv. *campestris* (Chan and Goodwin 1999, Sujatha et al. 2006). Among the identified virulence determinants, it has been demonstrated that the T3SS and the production of EPS as well as extracellular enzymes are independently essential for *X. campestris* pv. *campestris* to cause disease, thus representing two major virulence systems for the pathogen (Arlat et al. 1991; Chan and Goodwin 1999; Dow and Daniels 1994; Sujatha et al. 2006). In *Xanthomonas* spp., bacteria deliver many effector proteins into host cells by the T3SS, and the expression of T3SS-associated genes is under the positive control of HrpG, a response regulator be-

Table 3. Classification of genes whose expression was altered in the *rsmA_{Xcc}* mutant into functional categories based on sequence similarity to known genes

Functional category	Upregulated	Downregulated	Total
Amino acids biosynthesis	2	5	7
Biosynthesis of cofactors, prosthetic groups, carriers	1	1	2
Cell envelope	4	7	11
Cellular processes	16	31	47
Central intermediary metabolism	4	8	12
DNA metabolism	5	6	11
Energy metabolism	4	30	34
Fatty acid and phospholipid metabolism	1	2	3
Hypothetical proteins, conserved hypothetical proteins	88	118	206
Mobile and extrachromosomal element functions	7	1	8
Protein fate	3	9	12
Protein synthesis	4	1	5
Purines, pyrimidines, nucleosides, and nucleotides	1	0	1
Regulatory functions	10	11	21
Transcription	1	2	3
Transport and binding proteins	5	15	20
Unclassified	18	2	20
Unknown function	5	7	12
Total	179	256	435

longing to the OmpR family of two-component regulatory systems, and HrpX, an AraC-type transcriptional activator (Wengelnik and Bonas 1996; Wengelnik et al. 1996). Recently, another gene, *trh*, which encodes a putative transcriptional regulator, also has been shown to be involved in the regulation of T3SS (Tsuge et al. 2006). On the other hand, the synthesis of EPS and extracellular enzymes are positively regulated by *clp*, a gene encoding a CAP-like protein (de Crecy-Lagard et al. 1990), and a two-component regulatory system comprising the HD-GYP domain regulator RpfG and cognate sensor kinase RpfC (Barber et al. 1997; Dow et al. 2003; Slater et al. 2000; Tang et al. 1991). To our knowledge, *rsmA_{Xcc}* is the first regulator identified to control the two major virulence systems in *Xanthomonas* spp. This finding may provide a new clue for our understanding of the regulatory mechanisms of *X. campestris* pv. *campestris* pathogenicity.

In *P. aeruginosa*, RsmA positively regulates the production of extracellular protease and elastase (Pessi et al. 2001) whereas, in *Erwinia carotovora*, RsmA negatively regulates the production of the extracellular enzymes pectate lyase, cellulase, and protease (Chatterjee et al. 1995). Interestingly, in

X. campestris pv. *campestris*, *rsmA_{Xcc}* also positively controls the production of EPS besides extracellular enzymes (amylase and endoglucanase but not protease). To the best of our knowledge, this is the first observation that an *rsmA/csrA* regulates EPS production in bacteria. It has been proposed that, in *P. aeruginosa* and *E. carotovora*, RsmA regulates the production of extracellular products by modulating the quorum-sensing circuitry (Chatterjee et al. 1995; Pessi et al. 2001). In *X. campestris* pv. *campestris*, the quorum-sensing system also regulates the biofilm formation and the synthesis of EPS and extracellular enzymes, including amylase, endoglucanase, and protease (Dow et al. 2003). The overlap function of *rsmA_{Xcc}* and quorum-sensing system suggests that *rsmA_{Xcc}* also might regulate the production of extracellular products via modulating the quorum-sensing system in *X. campestris* pv. *campestris*. The microarray hybridization results showed that mutation in *rsmA_{Xcc}* did not alter the expression of genes known to be involved in the quorum-sensing system, such as *rpfCG* and *rpfF*, and production of EPS and extracellular enzymes, implying that *rsmA_{Xcc}* may affect these genes posttranscriptionally or by an unknown regulatory mechanism in *X. campestris*

Table 4. Virulence- and type III secretion system-associated genes whose expression was altered in the *rsmA_{Xcc}* mutant^z

ORF number	Gene name	Predicted product	Ratio of expression (<i>rsmA</i> -/wt)	RT-PCR results (<i>rsmA</i> - wt)
XC1553	<i>avr</i>	Leucine rich repeat protein	0.27	
XC2004	<i>avrXccC</i>	Avirulence protein	0.18	ND
XC2082	<i>avrBs1.1</i>	Avirulence protein	0.36	ND
XC2458	<i>manA</i>	Mannan endo-1,4-β-mannosidase	0.11	
XC2602	<i>avrXccE1</i>	Avirulence protein	0.45	ND
XC2827	<i>hpaR</i>	Transcriptional regulator marR family	0.49	
XC3001	<i>hpa2</i>	Hpa2 protein	0.15	
XC3002	<i>hpa1</i>	Hpa1 protein	0.03	
XC3003	<i>hrcC</i>	HrcC protein	0.12	
XC3005	<i>hrpB7</i>	HrpB7 protein	0.12	
XC3006	<i>hrpB6</i>	HrpB6 protein	0.35	
XC3008	<i>hrpB4</i>	HrpB4 protein	0.27	
XC3009	<i>hrcJ</i>	HrcJ protein	0.1	
XC3010	<i>hrpB2</i>	HrpB2 protein	0.06	
XC3011	<i>hrpB1</i>	HrpB1 protein	0.15	
XC3012	<i>hrcU</i>	HrcU protein	0.06	
XC3013	<i>hrcV</i>	HrcV protein	0.48	
XC3014	<i>hpaP</i>	HpaP protein	0.32	
XC3015	<i>hrcQ</i>	HrcQ protein	0.08	
XC3016	<i>hrcR</i>	HrcR protein	0.07	
XC3017	<i>hrcS</i>	HrcS protein	0.13	
XC3018	<i>hpaA</i>	HpaA protein	0.19	
XC3019	<i>hrpD5</i>	HrpD5 protein	0.06	
XC3020	<i>hrpD6</i>	HrpD6 protein	0.06	
XC3021	<i>hrpE</i>	HrpE protein	0.16	
XC3022	<i>hpaB</i>	HpaB protein	0.13	
XC3023	<i>hrpW</i>	HrpW protein	0.11	
XC3025	<i>hrpF</i>	HrpF protein	0.1	
XC3076	<i>hrpX</i>	HrpX protein	0.89	
XC3077	<i>hrpG</i>	HrpG protein	1.1	
Control: 16S rDNA	...	...	...	

^z ORF = open reading frame, wt = wild type, RT-PCR = reverse-transcription polymerase chain reaction, and ND = not done.

pv. *campestris*. It also is possible that the RpfCG quorum-sensing system regulates the expression or the function of *rsmA_{Xcc}*.

The *X. campestris* pv. *campestris* wild-type strain 8004 does not form cell aggregates in L medium but the *rsmA_{Xcc}* mutant does. The microarray hybridization and RT-PCR data showed that the expression of *manA* in the *rsmA_{Xcc}* mutant is significantly reduced (Table 4). It has been demonstrated that *manA* encodes the single endo- β -1,4-mannanase of *X. campestris* pv. *campestris* and the enzyme contributes to bacterial aggregate dispersal (Dow et al. 2003). These indicate that the enhanced cell aggregation of the *rsmA_{Xcc}* mutant may be due to the reduced expression of *manA*. However, how *rsmA_{Xcc}* affect the expression of *manA* remains to be further investigated.

A positive effect on the transcription of T3SS-associated genes by RsmA/CsrA has been reported in animal bacterial pathogens *S. enterica* serovar Typhimurium (Altier et al. 2000a and b; Lawhon et al. 2003) and *P. aeruginosa* (Mulcahy et al. 2006), and a plant bacterial pathogen, *E. carotovora* (Chatterjee et al. 2002). In *S. enterica* serovar Typhimurium and *P. aeruginosa*, in addition to the transcription of genes encoding the components of T3SS machinery, the transcription of the T3SS regulatory genes (*hilA*, *hilC*, and *hilD* in *Salmonella* spp. and *retS*, *exsA*, *exsC*, *cydB*, *vfr*, and *exsD* in *Pseudomonas* spp.) also is positively affected by RsmA/CsrA, indicating that, in these pathogens, RsmA/CsrA may regulate the T3SS regulatory genes indirectly (Lawhon et al. 2003; Mulcahy et al. 2006). In *E. carotovora*, HrpL_{Ecc} is required for the activation of *hrp* genes (Xiao and Hutcheson 1994). It has been demonstrated that RsmA promotes decay of *hrpL_{Ecc}* mRNA and thereby restricts the expression of HrpL_{Ecc}-dependent genes (Chatterjee et al. 2002). The microarray hybridization and RT-PCR data showed that the transcription of the two key regulatory genes of *X. campestris* pv. *campestris* T3SS, *hrpG* and *hrpX*, is not affected by *rsmA_{Xcc}*. The *X. campestris* pv. *campestris* wild-type strain and the *rsmA_{Xcc}* mutant strain are rifampicin resistant (Table 1); therefore, it is difficult to examine whether *rsmA_{Xcc}* promotes decay of *hrpG/hrpX* mRNAs. However, it is possible that the *X. campestris* pv. *campestris* RsmA_{Xcc} regulates the expression of *hrpG* and *hrpX* posttranscriptionally and then influences the expression of other *hrp*

genes regulated by HrpG/HrpX. Another possibility is that RsmA_{Xcc} regulates the transcription of *hrp* genes through an unknown regulatory pathway parallel to the HrpG/HrpX regulatory cascade. Works to verify these possibilities will be a subject for further studies.

All of the bacterial RsmAs/CsrAs identified so far are RNA-binding proteins. It has been demonstrated that the activity of RsmAs/CsrAs can be antagonized by regulatory small RNAs in *Escherichia coli* (Liu and Romeo 1997; Liu et al. 1997; Weilbacher et al. 2003), *Erwinia carotovora* (Cui et al. 1998; Liu et al. 1998), *P. aeruginosa* (Burrowes et al. 2005; Heurlier et al. 2004), *P. fluorescens* (Heeb et al. 2002; Kay et al. 2005; Valverde et al. 2003), *V. cholerae* (Lenz et al. 2005), and *S. enterica* (Altier et al. 2000b; Fortune et al. 2006). To further address the functional mechanism of RsmA_{Xcc}, it is important to identify the RsmA_{Xcc}-binding small RNAs. A search for sequences homologous to the identified RsmA/CsrA-binding small RNA-coding sequences revealed no similar sequences present in the genome of the *X. campestris* pv. *campestris* strain 8004. This result is not surprising because the RsmA/CsrA-binding small RNAs seem to be highly diversified, although the RsmA/CsrA proteins are highly conserved in different bacterial species. In *Escherichia coli*, for example, the small RNAs CsrB and CsrC show no sequence similarity to each other but both can interact with CsrA to antagonize CsrA (Liu and Romeo 1997; Weilbacher et al. 2003). Works to isolate the RsmA_{Xcc}-binding small RNAs in *X. campestris* pv. *campestris* are underway.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions.

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

Table 5. Predicted regulatory genes whose expression was altered in the *rsmA_{Xcc}* mutant^z

ORF number	Predicted product	Ratio of expression (<i>rsmA</i> ⁻ /wt)
Upregulated		
XC0136	RhsD protein truncated (RhsD)	10.20
XC0936	Methanol dehydrogenase regulatory protein	2.02
XC1260	Histidine kinase/response regulator hybrid protein	2.12
XC1474	RNA polymerase ECF-type sigma factor	2.39
XC2428	Ankyrin repeat protein	2.30
XC2592	RhsD protein	16.80
XC2840	Transcriptional regulator marR family (MarR)	4.73
XC3739	Tryptophan repressor binding protein	2.42
XC3801	Histidine kinase/response regulator hybrid protein	2.52
XC3997	Two-component system regulatory protein	2.45
Downregulated		
XC1290	Protein-glutamate methyltransferase (CheB)	0.20
XC1465	DNA methylation and regulatory protein Ada	0.34
XC2130	Single-domain response regulator (CheY)	0.41
XC2163	Protein-glutamate methyltransferase (CheB)	0.13
XC2578	Response regulator	0.30
XC2827	Transcriptional regulator marR family (HpaR)	0.49
XC3425	Transcriptional regulator (PcaQ)	0.45
XC3714	Sensor histidine kinase	0.31
XC3758	Regulatory protein (ExsF)	0.41
XC3966	Transcriptional regulator tetR family	0.44
XC4314	Sugar diacyl regulator	0.48

^z ORF = open reading frame and wt = wild type.

DNA manipulation.

DNA manipulation was performed following the procedures described by Sambrook and associates (1989). The conjugation between the *X. campestris* pv. *campestris* and *E. coli* strains was performed as described by Turner and associates (1985). Restriction enzymes and DNA ligase were used in accordance with the manufacturer's instructions (Promega, Shanghai, China).

Construction of an *rsmA_{xcc}* deletion mutant of *X. campestris* pv. *campestris*.

An *rsmA_{xcc}* deletion mutant of *X. campestris* pv. *campestris* was generated by marker exchange- eviction mutagenesis (Ried and Collmer 1987). A 762-bp upstream flanking sequence (designated as FL) (before the start codon [ATG]) and an 895-bp downstream flanking sequence (designated as FR) (after the stop codon [TGA]) of the *rsmA_{xcc}* gene of *X. campestris* pv. *campestris* were amplified by PCR using the total DNA of the wild-type strain 8004 as the template and the primer pairs 2506DLF/2506DLR and 2506DRF/2506DRR, respectively. After being confirmed by sequencing, the FL fragment was digested with *Bam*HI and *Eco*RI and the FR fragment was digested with *Eco*RI and *Hind*III, and the two digested fragments and the *Bam*HI- and *Hind*III-digested DNA of the suicide plasmid pKsacB were linked together by T4-DNA ligase to generate the recombinant plasmid named pKrsmAFL in *E. coli* JM109 (Table 1). The plasmid pKrsmAFL was transferred from *E. coli* into the *X. campestris* pv. *campestris* wild-type strain 8004 by triparental conjugation using pRK2073 (Leong et al. 1982) as a helper. The integration transconjugants (integrated the pKrsmAFL into the chromosome of the strain 8004 by single-crossover) were screened on NYG agar plates containing rifampicin and kanamycin. To generate an *rsmA_{xcc}* deletion mutant from the integration transconjugants, overnight culture of a sucrose-sensitive integration transconjugant was diluted and plated onto NYG plates containing rifampicin and 5% sucrose. After incubation at 28°C for 3 days, the rifampicin- and sucrose-resistant clones were picked up and tested for kanamycin sensitivity. Total DNA was isolated from the rifampicin- and sucrose-resistant but kanamycin-sensitive clones and screened by PCR using primer pair 2506DLF/2506DRR to identify the clones which had lost the chromosomal *rsmA_{xcc}*-coding region after double-crossover. Of 40 rifampicin- and sucrose-resistant but kanamycin-sensitive clones tested, 12 were *rsmA_{xcc}* deleted. One of the confirmed mutants was named DM2506 (Table 1) and chosen for further study.

Complementation of the *rsmA_{xcc}* deletion mutant DM2506.

For complementation of the *rsmA_{xcc}* deletion mutant DM2506, a 512-bp DNA fragment containing the entire *rsmA_{xcc}* gene (from 200 bp upstream of the start codon to 100 bp downstream of the stop codon) was amplified by PCR using the total DNA of the *X. campestris* pv. *campestris* wild-type strain 8004 as the template and the primer pair 2506CF/2506CR. After being confirmed by sequencing, the amplified DNA fragment was cloned into pLAFR3 to generate the recombinant plasmid named pLrsmA_{xcc} (Table 1). The plasmid pLrsmA_{xcc} was transferred into the mutant DM2506 by triparental conjugation (Turner et al. 1985). The transconjugants carrying pLrsmA_{xcc} were screened on NYG plates with rifampicin and tetracycline. A confirmed transconjugant representative was named CDM2506 (Table 1) and chosen for further study.

Competitive growth assay.

An overnight culture (1 ml, optical density at 600 nm [OD₆₀₀] ≈ 1.0) mixture (1:1) of the *rsmA_{xcc}* mutant strain and the wild-type strain was inoculated into 100 ml MMX minimal

medium in a 250-ml flask and incubated at 28°C with 200 rpm. At the time points of 56 and 72 h (exponential and stationary phase, respectively) postinoculation, the cell number of the *rsmA_{xcc}* mutant strain and the wild-type strain in the culture was determined using dilution plate counting method, and their CFU were counted and compared. The *rsmA_{xcc}* mutant is EPS deficient and forms small, dry colonies which are distinguished from those of the wild type. Each experiment was performed at least in triplicate.

Test of extracellular enzyme activity and EPS production.

The activity of the extracellular enzymes amylase, endoglucanase, and protease was estimated quantitatively using the following experimental procedures. For endoglucanase, 10 µl of cytoplasmatic extract was added to 200 µl of indicated buffer containing 1% (wt/vol) CMC. Reactions were carried out for 30 min at 28°C. The released reducing sugars were measured as D-glucose equivalents as described by Miller (1959). One unit (U) of the endoglucanase activity was defined as the amount of enzyme releasing 1 µmol of reducing sugar per minute. Amylase activity quantification was conducted the same as endoglucanase measurement, except that the substrate 1% (wt/vol) CMC was replaced by 1% (wt/vol) starch solution. The quantification of protease activity was done as described by Swift and associates (1999).

The EPS production was measured as described by Wilson and associates (1998) with a minor modification. Overnight culture (2 ml, OD₆₀₀ ≈ 1.0) of each *X. campestris* pv. *campestris* strain was used to inoculate 100 ml of NYG medium containing 4% glucose in a 500-ml flask and kept at 28°C with shaking at 200 rpm for 5 days. The EPS was precipitated from the culture supernatant by the addition of three volumes of 95% ethanol. The pelleted EPS was washed with 70% ethanol, air dried, and weighed. Three flasks were inoculated in each experiment, and each experiment was repeated three times.

LPS analysis.

LPS was prepared using an LPS extraction kit (iNtRON Biotechnology, Korea). LPS preparations were analyzed using Tricine sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (Lesse et al. 1990) and visualized by silver staining (Kittelberger and Hilbink 1993).

Virulence assay and determination of bacterial load in planta.

Virulence of the *X. campestris* pv. *campestris* strains was tested on potted Chinese radish (*R. sativus* L. var. *radiculus* Pers.) growing in a greenhouse with a day-and-night cycle of 12-and-12 h with illumination by fluorescent lamps at 25 to 28°C. Seedlings with four fully expanded leaves were used for inoculation. Bacterial cells were grown in NYG medium at 28°C with shaking at 200 rpm for 15 h (at the exponential phase of growth). The concentration of bacterial inocula was adjusted to an OD₆₀₀ of 0.1. Two leaves per plant were inoculated by the leaf-clipping method (Dow et al. 2003). Sixty leaves were inoculated for each independent experiment, which was repeated three times. After maintenance in 100% humidity for 24 h, the inoculated plants were maintained in the growth conditions described above. Lesion length was measured 10 days postinoculation. The same experiment was repeated three times.

The growth of bacteria in infected leaf tissues was measured using the method previously described (Tang et al. 2005).

HR test.

The HR was tested on the pepper plant ECW-10R (*Capsicum annuum* cv. ECW-10R), which is one of the nonhosts

commonly used to test the HR of *X. campestris* pv. *campestris* (Castañeda et al. 2005; Newman et al. 2001). The plants were inoculated by infiltrating approximately 5 µl of bacterial culture (1×10^8 CFU/ml) suspended in 10 mM sodium phosphate buffer (5.8 mM Na_2HPO_4 and 4.2 mM NaH_2PO_4 , pH 7.0) into the abaxial leaf surface using a blunt-end plastic syringe. The inoculated plants were maintained in a greenhouse with a 12-h day-and-night cycle with illumination by fluorescent lamps and a constant temperature of 28°C, and were observed at 8, 12, 24, 36, and 48 h after inoculation. HR symptoms were photographed 48 h postinoculation. At least three plants were inoculated in each experiment, and each experiment was repeated at least twice.

Motility assay.

To test the motility of the *X. campestris* pv. *campestris* strains, 2 µl of overnight culture ($\text{OD}_{600} \approx 1.0$) of each strain was spotted onto NYG plates containing 2% (wt/vol) glucose and 0.3% (wt/vol) agar and incubated at 28°C for 3 days.

Cell aggregation assay.

Overnight culture (300 µl, $\text{OD}_{600} \approx 1.0$) of *X. campestris* pv. *campestris* strains was inoculated into 5 ml of L medium in a 25-ml universal bottle (Sangon, Shanghai, China) and incubated steadily at 28°C for 4 days. Bacterial cell aggregates formed on the surface of the medium or attached to the bottle wall were surveyed. Each experiment was performed at least in triplicate.

Adhesion assay.

Quantitative analysis of adhesion of *X. campestris* pv. *campestris* cells on glass surface was tested using the method described by Jackson and associates (2002). Overnight culture (100 µl, $\text{OD}_{600} \approx 1.0$) of each strain was inoculated into 5 ml of NYG liquid medium in a 25-ml universal tube (Sangon) and incubated steadily at 28°C for 24 h. The cells that bound to the glass surface were measured by discarding the medium, rinsing the tubes with water (three times), and staining bound cells with crystal violet (Sangon). The dye was solubilized with 33% acetic acid (Sangon) and absorbance at 630 nm was determined using an Ultrospec 3300 pro spectrophotometer (Amphamacia Biotech, Biochrom Ltd, Cambridge, U.K.). Each experiment was performed at least in triplicate.

Glycogen accumulation test.

To test glycogen accumulation in *X. campestris* pv. *campestris* cells, 2 µl of overnight culture ($\text{OD}_{600} \approx 1.0$) of each testing strain was spotted onto NYG plates containing 2% (wt/vol) glucose, and incubated at 28°C for 4 days. The plates were inverted and placed over a 500-ml beaker containing 3.3% iodine–6.6% potassium iodide solution. The colonies were stained with the iodine vapor by heating the solution for 3 to 5 min. Colonies in which glycogen had accumulated were dark brown in color, while those with little or no glycogen production stained yellow.

Construction of the DNA microarray.

The genome of the *X. campestris* pv. *campestris* strain 8004 (GenBank accession number CP000050) is composed of 4,273 ORF (Qian et al. 2005). A DNA microarray encompassing 4,186 ORF of the strain 8004 has been constructed previously as described by He and associates (2007) and used in this study.

RNA isolation and cDNA synthesis.

For RNA isolation, 2 ml of an overnight cell culture of the *X. campestris* pv. *campestris* wild-type strain 8004 or the

rsmA_{Xcc} mutant DM2506 was diluted into 100 ml of MMX medium and grown at 28°C with shaking at 200 rpm. When the cells reached an OD_{600} of 1.2, cells were collected by centrifugation for 2 min at $5,000 \times g$. The pellets were resuspended in 10 ml Tris-EDTA buffer (pH 8.0) and frozen in a liquid nitrogen bath for 15 min. Total RNA was extracted with TRIzol (Invitrogen, San Diego, CA, U.S.A.) and the contaminated DNA was removed by RQ DNase I (Promega) treatment. RNA integrity was confirmed by electrophoresis using a 1.3% formaldehyde-agarose gel, and the RNA quality was monitored by RT-PCR analysis of at least two known genes. RNA was used for cDNA synthesis or stored at –80°C until further use.

The RNAs isolated from the wild-type strain 8004 and the *rsmA_{Xcc}* mutant DM2506 were used to make both Cy3- and Cy5-labeled cDNAs by reverse transcription and Klenow enzyme reaction. Briefly, 20 µg of total RNA and a series of dilutions (from 1 ng to 20 pg) of yeast intergenic spike RNA were utilized in reverse transcription reaction using 3 µg of nonamer random primer and 200 units of M-MLV transcriptase (Invitrogen) in $1\times$ first buffer at 37°C for 1 h. After the completion of the reaction, RNA was hydrolyzed by adding 5 µl of stop buffer (35 mM EDTA, 1.4 M NaOH) and neutralized with 1 µl of acetic acid (10 N). cDNA was purified with PCR Clean-up NucleoSpin Extract II kits (Macherey-Nagel, Düren, Germany) and dry vacuumed.

Slide hybridization and data analysis.

For expression profiling hybridization, the labeled control and test cDNA samples were quantitatively adjusted according to the efficiency of Cy-dye incorporation, and then mixed with 80 µl of hybridization solution ($3\times$ SSC [$1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.2% SDS, and 50% formamide). Prior to loading on the microarray, the mixture was heated to 95°C for 3 min to denature the cDNA in hybridization solution. Hybridization was performed under LifterSlip (Erie Company, Portsmouth, NH, U.S.A.), which allows for an even dispersal of hybridization solutions between the microarray and coverslip. The hybridization chamber was laid on a three-phase Tiling Agitator (CapitalBio Corp., Beijing) to prompt the microfluidic circulation under the coverslip. The array was hybridized at 42°C overnight and washed with two consecutive washing solutions (0.2% SDS, $2\times$ SSC) at 42°C for 5 min and 0.2% SSC for 5 min at room temperature.

Arrays were scanned with a confocal LuxScan scanner (CapitalBio Corp.), and signal intensities were detected and quantified with SpotData software (CapitalBio Corp.) and assembled into EXCEL spreadsheets. Expression ratios were calculated as *rsmA_{Xcc}* mutant/wild type (*rsmA_{Xcc}*/WT), and the mean, standard deviation, and statistical significance of the expression difference was calculated following the published method (Yang et al. 2003). Measurements with high variability (those where the standard deviation was equal to or larger than the mean) or those where valid ratios were found for only one of the three replicates were removed. The microarray hybridization was repeated twice.

Semiquantitative RT-PCR analysis.

To confirm the microarray data, semiquantitative RT-PCR analysis was carried out. RNA was isolated from cultures of the *X. campestris* pv. *campestris* wild-type strain 8004 and the *rsmA_{Xcc}* mutant DM2506 grown to an OD_{600} of 1.0 in the MMX medium. The same amount of RNA of the wild-type strain 8004 and the mutant DM2506 was used to convert to cDNA using a TaKaRa RNA PCR Kit (AMV; version 3.0; TaKaRa Biotechnology Co., Ltd. Dalian, China). The cDNAs were used as the templates for amplification in PCR with *Taq*

polymerase, and the primers used were listed in Supplemental Table S2. PCR was performed with a cycler using the following cycle parameters: 30 cycles of 94°C for 15 s, 60°C for 15 s, and 72°C for 15 s. The resulting amplification products were analyzed in 1.2% agarose gels. The 16S rRNA gene of *X. campestris* pv. *campestris* was used as the internal control to verify the absence of significant variation at cDNA level in the two samples.

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