Identification and Characterization of Two Novel DSF-Controlled Virulence-Associated Genes Within the *nodB-rhgB* Locus of *Xanthomonas oryzae* pv. *oryzicola* Rs105

Zhiwei Song, Yancun Zhao, Xingyang Zhou, Guichun Wu, Yuqiang Zhang, Guoliang Qian, and Fengquan Liu

First, third, fourth, fifth, sixth, and seventh authors: College of Plant Protection, Nanjing Agricultural University, Nanjing 210095, China/Key Laboratory of Integrated Management of Crop Diseases and Pests (Nanjing Agricultural University), Ministry of Education, China; and second and seventh authors: Institute of Plant Protection, Jiangsu Academy of Agricultural Science, Nanjing 210014, P.R. China.
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ABSTRACT

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Xanthomonas oryzae pv. *oryzicola* and *X. oryzae* pv. *oryzae* are two pathovars of *X. oryzae* that cause leaf streak and blight in rice, respectively. These two bacterial pathogens cause different disease symptoms by utilizing different infection sites on rice. Compared with *X. oryzae* pv. *oryzae*, the molecular virulence mechanism of *X. oryzae* pv. *oryzicola* remains largely unknown. Previously, we identified a unique diffusible signal factor (DSF)-controlled virulence-related gene (*hshB*) in *X. oryzae* pv. *oryzicola* Rs105 located in the *nodB-rhgB* locus, which is absent in *X. oryzae* pv. *oryzae* PXO99A. In the present study, we identified two additional genes within this locus (*hshA* and *hshC*) that were unique to *X. oryzae* pv.

oryzicola Rs105 compared with *X. oryzae* pv. *oryzae* PXO99A, and we found that the transcription of these genes was regulated by DSF signaling in *X. oryzae* pv. *oryzicola*. The mutation of these genes impaired the virulence of the wild-type Rs105 when using a low inoculation density of *X. oryzae* pv. *oryzicola*. In contrast to *hshB*, the mutation of these genes did not have any visible effect on characterized virulence-related functions, including in vitro growth, extracellular polysaccharide production, extracellular protease activity, and antioxidative ability. However, we found that mutation of *hshA* or *hshC* significantly reduced the in planta growth ability and epiphytic survival level of *X. oryzae* pv. *oryzicola* cells, which was the probable mechanisms of involvement of these two genes in virulence. Collectively, our studies of *X. oryzae* pv. *oryzicola* have identified two novel DSF-controlled virulence-associated genes (*hshA* and *hshC*), which will add to our understanding of the regulatory mechanisms of conserved DSF virulence signaling in *Xanthomonas* species.

Xanthomonas is a large genus of gram-negative bacteria with a typical yellow appearance in colonies. This genus consists of a large number of phytopathogenic bacteria, which infect diverse crop plants of economic significance, including rice, citrus, banana, cabbage, and bean (22,25). *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* are two pathovars of *X. oryzae*. These two pathovars utilize distinct mechanisms to infect rice leaves and cause different symptoms. *X. oryzae* pv. *oryzae* is a vascular pathogen that enters rice leaves via the hydathodes causing bacterial blight, whereas *X. oryzae* pv. *oryzicola* penetrates rice leaves mainly through stomata or wound sites and colonizes intercellular spaces in the mesophyll, resulting in bacterial leaf streak (4). Compared with *X. oryzae* pv. *oryzae*, the molecular virulence mechanism of *X. oryzae* pv. *oryzicola* is poorly understood. Therefore, discovery of genetic determinants of virulence in *X. oryzae* pv. *oryzicola* will aid us to address this point.

Quorum sensing (QS) is a chemical signal-dependent and widely conserved cell-cell communication mechanism that coordinates various bacterial behaviors and gene expression patterns in a cell density-dependent manner (15). In most gram-negative bacteria, QS is mediated by the production and perception of *N*-acyl homoserine lactones (AHLs) by two paired proteins belonging to the LuxI and LuxR families (11). However, *Xanthomonas* species

do not produce AHLs; instead, they produce another type of QS signal, a diffusible signal factor (DSF). This signal was initially identified from *X. campestris* pv. *campestris* as *cis*-11-methyl-2-dodecenoic acid (1,24). Recently, DSF and DSF-like molecules have been shown to be widely distributed in plant-associated bacteria, including *Xanthomonas*, *Burkholderia*, and *Xylella* (3,6). The regulation of pathogenicity factor (*rpf*) gene cluster is required for DSF signaling in *Xanthomonas* (2,14). The gene *rpfF* encodes a putative enoyl-CoA hydratase that catalyzes the biosynthesis of the DSF molecule, while the two-component system RpfC-RpfG is responsible for the sensing and transduction of DSF signals (1,13). Just like AHLs, DSF signals play important roles in virulence, cell motility, biofilm dispersal, the activity of extracellular enzymes, and extracellular polysaccharide (EPS) production in diverse bacteria (5,8,10,14). Previously, we showed that the DSF cell-cell signaling of *X. oryzae* pv. *oryzicola* is required for virulence on host rice (27). A subsequent proteomic analysis of the wild-type strain Rs105 and a DSF-deficient mutant allowed us to identify several novel and DSF-controlled virulence factors in *X. oryzae* pv. *oryzicola*; one of them is encoded by *hshB* (*XOC_3772*), located in the *nodB-rhgB* locus of *X. oryzae* pv. *oryzicola* (26). This locus consists of 10 genes; four of them (*hshB*, *hshA*, *XOC_3770*, and *XOC_3769*) are genetically adjacent and predicted to be unique to the genome of *X. oryzae* pv. *oryzicola* Rs105 compared with *X. oryzae* pv. *oryzae* PXO99A (26). To be consistent with the names *hshA* and *hshB*, we hereafter refer to *XOC_3770* and *XOC_3769* as *hshC* and *hshD*, respectively. With the exception of *hshB*, the roles of the three remaining genes (*hshA*, *hshC*, and *hshD*) in virulence as well as their relationships with DSF signaling in *X. oryzae* pv. *oryzicola* are unknown.

Here, we provide experimental evidence showing that the homologs of *hshA*, *hshC*, and *hshD* of *X. oryzae* pv. *oryzicola* Rs105 were lacking

Corresponding authors: F. Liu; E-mail address: fqliu20011@sina.com and G. Qian; E-mail address: glqian@njau.edu.cn

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in *X. oryzae* pv. *oryzae* PXO99^A. The transcriptions of *hshA* and *hshC*, but not *hshD*, were found to be regulated by the DSF system in *X. oryzae* pv. *oryzicola*. We further found that mutation of *hshA*, *hshC*, or double genes in *X. oryzae* pv. *oryzicola* caused a significant reduction of virulence on host rice when the inoculated cell population was low. Finally, we show that the decrease of *hshA* and *hshC* in virulence was associated with the reduced in planta cell colonization ability and the epiphytic survival level of the corresponding mutants. Collectively, our results provide two novel virulence-related genes (*hshA* and *hshC*) in *X. oryzae* pv. *oryzicola*, which is important for understanding the molecular mechanism of *X. oryzae* pv. *oryzicola* virulence.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this work are listed in Supplementary Table 1. *Escherichia coli* strains were cultivated at 37°C in Luria-Bertani (LB) broth or LB agar plates. Unless specified otherwise, the wild-type strain Rs105 of *X. oryzae* pv. *oryzicola* and its derivative strains were grown at 28°C in nutrient broth (NB) medium (beef extract, 3 g/liter; yeast extract, 1 g/liter; polypeptone, 5 g/liter; and sucrose 10 g/liter) or on nutrient agar (NA). MMX (glucose at 5 g/liter, [NH₄]₂SO₄ at 2 g/liter, MgSO₄ · 7H₂O at 0.2 g/liter, K₂HPO₄ at 4 g/liter, KH₂PO₄ at 6 g/liter, and trisodium citrate at 1 g/liter, pH 7.0) was used as the minimal medium. When required, antibiotics were added to the medium at the following final concentrations: ampicillin (Ap) at 100 µg/ml and kanamycin (Km) at 50 µg/ml for *E. coli*; rifampicin (Rif) at 100 µg/ml and Km at 50 µg/ml for *X. oryzae* pv. *oryzicola*.

Generation of gene deletion mutants and complemented strains. The *X. oryzae* pv. *oryzicola* wild-type Rs105 was used as the parental strain for generating in-frame deletion mutants via allelic homologous recombination, as described previously (17,27). In brief, two flanking regions of each gene were generated by polymerase chain reaction (PCR) amplification by using different primer pairs. Two corresponding fragments of each gene were digested with appropriate restriction enzymes, and cloned into suicide vector pK18mob*sacB* (28), creating individual recombinant vectors for each selected gene. These constructions were transformed into the wild-type strain Rs105 by electroporation, respectively. Transconjugants were selected on NA plates without sucrose and with Rif (100 µg/ml) and Km (50 µg/ml). Positive colonies were plated on NA plates containing 10% (wt/vol) sucrose and Rif (100 µg/ml) to select for resolution of the construct by a second cross-over event. The resultant mutant containing the gene in-frame deletion was confirmed by PCR and Southern blotting (data not shown). For complementation, a series of expected DNA fragments containing each intact gene and predicted promoter were amplified by PCR method by using the primers listed in Supplementary Table 1 and cloned into the complemented-vector pUFR034 (7). The resultant constructs were transferred into the corresponding mutants by electroporation and confirmed by PCR, as described previously (17,27).

Southern blot hybridization. Southern blot analysis was performed to verify the *hshA*, *hshC*, and *hshD* genes are unique in *X. oryzae* pv. *oryzicola* Rs105 compared with *X. oryzae* pv. *oryzae* PXO99^A. For each of the three genes, two probes were used. Probe P1 is the internal fragment of each of the tested three genes. Probe P2 is the internal fragment of a conserved gene (*grx*, located in *nodB-rghB* locus) both in *X. oryzae* pv. *oryzicola* Rs105 and *X. oryzae* pv. *oryzae* PXO99^A. Three probe pairs, including P1-*hshD* and P2, P1-*hshC* and P2, and P1-*hshA* and P2, were used to hybridize the genomic DNA of *X. oryzae* pv. *oryzicola* Rs105 and *X. oryzae* pv. *oryzae* PXO99^A. In brief, a 533-bp internal fragment of *hshD* was amplified by primers *hshD*-F/*hshD*-R and labeled as the probe P1-*hshD*; a 519-bp internal fragment of *hshC* was amplified by primers *hshC*-F/*hshC*-R and labeled as the probe

P1-*hshC*; a 710-bp internal fragment of *hshA* was amplified by primers *hshA*-F/*hshA*-R and labeled as the probe P1-*hshA*. Before hybridization, the genomic DNA of *X. oryzae* pv. *oryzicola* Rs105 and *X. oryzae* pv. *oryzae* PXO99^A was digested by appropriate restriction enzymes (Fig. 1A). In brief, in the assay involving in *hshD*, the genomic DNA of *X. oryzae* pv. *oryzicola* Rs105 was digested by *Bam*HI and *Pst*I. In the assay involved in *hshC* and *hshA*, the genomic DNA of *X. oryzae* pv. *oryzicola* Rs105 was digested by *Bam*HI and *Xba*I. In all experiment, the genomic DNA of *X. oryzae* pv. *oryzae* PXO99^A was digested by *Bam*HI and *Hind*III. All these digested genomic DNA samples were then separated by electrophoresis in a 1% agarose gel. The following steps of DNA hybridization were performed as described previously (18).

Pathogenicity test and determination of bacterial load in rice plants. Pathogenicity assays were performed in a glasshouse at 22 to 30°C as previously described (17,27). In brief, *X. oryzae* pv. *oryzicola* strains were cultivated in NB medium at 28°C with appropriate antibiotics. Each *X. oryzae* pv. *oryzicola* strain was adjusted to a series of cell concentrations (OD_{600nm} = 0.5, 0.05, 0.005, and 0.0005, respectively). Then, these cell suspensions were infiltrated into the leaves of 2-week-old rice seedlings (Shanyou-63, susceptible to the pathogen) by needless syringe. The water-soaking symptoms were measured at 10 days after inoculation. Ten leaves were inoculated for each *X. oryzae* pv. *oryzicola* strain in each treatment. The same experiment was performed three times.

Determination of bacterial load in infected rice plants was performed as described previously (26). In brief, colonization of each *X. oryzae* pv. *oryzicola* strain in rice leaf tissue was detected by homogenizing five inoculated leaves in 9 ml of sterile water. The leaves were cut in 6-mm sections around the inoculation spots after 5 days of inoculation, while the same process was used to deal with leaves after 10 days of inoculation. Diluted homogenates were plated on NA plates supplemented with Rif (for the wild type and mutants). The number of bacterial colonies on these plates was counted after 2 days of incubation at 28°C. Each diluted homogenate was plated on three plates, respectively. Three replicates for each treatment were used, and the experiment was performed three times.

Determination of bacterial epiphytic survival on rice leaf surface. The *X. oryzae* pv. *oryzicola* epiphytic survival assay was performed according to a recent report about *X. axonopodis* pv. *citrius* (21), with minor modification. In brief, the cell population (10⁸ CFU/ml) of the wild-type Rs105 and its derivatives were sprayed onto the leaves of 2-week-old rice seedlings (Shanyou-63, susceptible to the pathogen). After spraying, at least 10 rice leaves treated by each strain were collected at various time points (0.5, 4, 12, 20, and 28 h), respectively. Then, these rice leaves were cut into 6-mm sections, following by washing three times with sterile ddH₂O to release the bacterial cells that were not adhered to leaf surface. Next, these pretreated rice leaf sections were homogenized, and diluted homogenates were plated on NA plates supplemented with Rif (for the wild type and mutants). The number of bacterial colonies on these plates was counted after 2 to 3 days of incubation at 28°C. Each diluted homogenate was plated on three plates, respectively. Three replicates for each treatment were used, and the experiment was performed three times.

To statistically calculate the epiphytic survival level of each strain throughout the 28 h, we used an AUPDC methodology (area under population dynamics curve) to do this analysis. The AUPDC analysis was performed as described previously (2), and it was calculated as follows:

$$\text{AUPDC} = \sum_{i=1}^n [(Y_{i+1} + Y_i)/2](X_{i+1} - X_i)$$

where Y_i is the log₁₀ bacterial population at the i th observation, X_i is the time in hours at the i th observation, and n is the total number of observations. The AUPDC value of each strain was used to

determine the significance between wild-type Rs105 and each tested mutant in the present study.

Growth rate of *X. oryzae* pv. *oryzicola* strains in MMX and NB. *X. oryzae* pv. *oryzicola* strains were grown in NB broth at 28°C with shaking at 200 rpm. Cells were collected at the early log phase ($OD_{600nm} = 0.5$) by centrifugation at $3,000 \times g$. Cell pellets were suspended in an equal volume of sterile ddH₂O. Then, 0.5 ml of cell suspension was inoculated into 100 ml of NB (a nutrient-rich broth). All inoculation broths were grown at 28°C with shaking at 200 rpm, and the OD_{600nm} value was determined every 8 h until bacterial growth reached the stationary stage. The growth of *X. oryzae* pv. *oryzicola* strains in MMX (a minimal medium) was also measured. In brief, the initial inoculated bacterial concentration of tested strains was adjusted to $OD_{600nm} = 1.0$. Then, a 300- μ l suspension was added into 30 ml of MMX at 28°C with shaking at 200 rpm, and the OD_{600nm} value was determined every 12 h. Three replicates for each treatment were used, and the experiment was repeated three times.

Resistance to hydrogen peroxide. Experiments testing resistance to hydrogen peroxide (H₂O₂) were carried out as described previously (26). In brief, the NA plates were made containing H₂O₂ concentrations of 0, 0.1, and 0.25 mM. *X. oryzae* pv. *oryzicola* strains were cultured to midlogarithmic phase ($OD_{600nm} = 1.0$) in NA medium. Then, threefold and ninefold dilutions were made. An aliquot of 5 μ l of initial culture and diluted cultures for each strain were spotted on NA plates and cultured for 36 h at 28°C. Each strain was repeated three times. The same experiment was repeated at least three times.

Quantification of EPS production. EPS production of *X. oryzae* pv. *oryzicola* strains was measured with some modification as described previously (23,26). Briefly, *X. oryzae* pv. *oryzicola* strains were grown in NB broth containing 4% glucose at 28°C with shaking at 200 rpm for 5 days. EPS was precipitated from the culture supernatant with ethanol and dried at 80°C to constant weight. The difference between the two weights was used to estimate the production of EPS per milliliter of culture. Every experiment was repeated three times, and each treatment involved at least three replicates.

Determination of extracellular protease activity of *X. oryzae* pv. *oryzicola* strains. To measure the extracellular activity of protease, 2 μ l of bacterial culture ($OD_{600nm} = 1.0$) was spotted on NB agar plates containing 1% (wt/vol) skim milk powder. After incubation at 28°C for 36 h, protease activity was measured and compared according to the hydrolytic zones around bacterial colony (21,26). Each strain was repeated three times, and the same experiment was repeated at least three times.

Determination of cell swimming motility. Cell swimming motility assays were performed as described previously (19). In brief, cell cultures of each strain were normalized to an OD_{600nm} of 0.5, and 3 μ l of each prepared cultures was inoculated onto the surface of the motility plates (NA containing 0.3% agar). The motility halo was examined at 4 days after incubation at 28°C. Three replicates for each treatment were used, and the experiment was performed three times.

DSF extraction and detection. DSF extraction from *X. oryzae* pv. *oryzicola* was performed as described previously (16,26). Briefly, the *X. oryzae* pv. *oryzicola* wild-type Rs105 was cultured to an OD_{600nm} of 3.0 (stationary phase) in 200 ml of NB broth at 28°C with shaking at 200 rpm. The culture was centrifuged at 8,000 rpm for 20 min at 4°C. Then, the supernatant was collected into a separating funnel, following by addition of 200 ml of ethyl acetate for DSF signal extraction. The ethyl acetate phase was concentrated 100-fold by evaporation, and was finally dissolved in 1 ml of dimethyl sulfoxide. The assay for determination of the activity of DSF extraction was performed as described previously, using a DSF biosensor (16,27). The validated DSF signal was used for further studies (see below).

Real-time PCR. Quantitative real-time reverse transcription PCR (qRT-PCR) was carried out by using SYBR Premix EX TagTM

II kit (TaKaRa) in an ABI PRISM 7500 Real-Time PCR System (Applied Biosystems). The 16S rRNA was used as an endogenous control. Primer sequences used in this assay were listed in Supplementary Table 2. All the *X. oryzae* pv. *oryzicola* strain were grown in NB broth at 28°C with shaking at 200 rpm. For DSF supplement, 125 μ l of the validated DSF extraction was added into 25 ml of the culture of the *rpfF* mutant (the DSF-deficient mutant) (26). The cells of each strain, including those of the *rpfF* mutant treated by DSF extraction, were collected at the midlogarithmic phase ($OD_{600nm} = 1.0$). These cells were used for RNA extraction using the RNAiso Plus reagent (TaKaRa) following the manufacturer's instructions. To remove genomic DNA, the eluted RNA samples were treated with RNase inhibitors and DNaseI (TaKaRa). RNA integrity was confirmed by electrophoresis using 1.2% agarose gels. Then 2 μ g of each RNA sample was used to synthesize cDNA with a cDNA synthesis kit (TaKaRa). The same experiment was performed three times. In this study, the transcriptional level of *hshD*, *hshC*, and *hshA* in wild-type Rs105 and its derivatives was assessed and compared, respectively. The gene *hshB* was used as a positive control; this gene was known to be regulated by DSF system at transcriptional level (26).

Data analysis. All analyses were conducted using SPSS 14.0 (SPSS Inc., Chicago, IL). The hypothesis test of percentages (*t* test, *P* = 0.05) was used to determine significant differences in the pathogenicity test, in planta colonization, epiphytic survival, and gene expressions.

RESULTS

Sequence analysis of the *nodB-rhgB* locus in *Xanthomonas* and related species. In the available genome of *X. oryzae* pv. *oryzicola* BLS256, the *nodB-rhgB* locus consists of 10 genes. This locus was highly conserved in *X. oryzae* pv. *oryzicola* Rs105 (a Chinese isolate) used in this study (26). A BlastP search indicated that this locus was relatively conserved in *Xanthomonas*. As shown in Table 1, two or three of the genes (*nodB*, *grx*, and *rhgB*) were highly conserved in *Xanthomonas* and related species such as *Stenotrophomonas maltophilia* R551-3 (a nosocomial pathogen) and *Xylella fastidiosa* 9a5c (the pathogen of Pierce's disease in grape). Sequence characterization of the genes within this locus showed that *nodB* encodes acetylxylin esterase; *grx* encodes a hypothetical protein; and *rhgB* encodes rhamnogalacturonase B in the published genomes of *Xanthomonas*.

Similar to *hshB*, three other genes (*hshA*, *hshC*, and *hshD*) in this locus were not conserved in *Xanthomonas* and related species. The gene *hshC* encodes a hypothetical protein; *hshA* encodes an amidohydrolase family protein whose amidohydrolase family domains were similar to those of HshB (Supplementary Fig. 1); and *hshD* encodes a product belonging to the appr-1-p processing enzyme family. A detailed analysis revealed that different *Xanthomonas* species contained different numbers of these four genes at this locus. For example, *X. campestris* pv. *vesicatoria* 85-10 (the pathogen causing bacterial spot of pepper/tomato) contained homologs of these three genes (*hshA*, *hshB*, and *hshC*), and their organization in the genome was the same as that found in *X. oryzae* pv. *oryzicola*. *X. campestris* pv. *campestris* ATCC33913 (the pathogen causing black rot of crucifers) also harbored homologs of these three genes (*hshA*, *hshB*, and *hshC*), while *X. axonopodis* pv. *citri* 306 (the pathogen causing citrus canker) had only one gene homolog (*hshC*). Interestingly, although *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola*, belonging to two pathovars of *X. oryzae*, shared a number of conserved proteins/genes, the homologs of three genes (*hshA*, *hshB*, and *hshC*) were all missing from the published genomes of *X. oryzae* pv. *oryzae* PXO99A, *X. oryzae* pv. *oryzae* MAFF311018, and *X. oryzae* pv. *oryzae* KACC10331, three model *X. oryzae* pv. *oryzae* strains. More interestingly, the *hshD* homolog was detected

in the genomes of *X. oryzae* pv. *oryzae* MAFF311018 and *X. oryzae* pv. *oryzae* KACC10331, but not in *X. oryzae* pv. *oryzae* PXO99^A genome.

Southern blotting verified the uniqueness of *hshA*, *hshC*, and *hshD* in the *X. oryzae* pv. *oryzicola* Rs105 genome compared with *X. oryzae* pv. *oryzae* PXO99^A. As noted above, four genes (*hshA*, *hshB*, *hshC*, and *hshD*) in the *nodB-rhgB* locus of *X. oryzae* pv. *oryzicola* Rs105 were predicted to be lacking in *X. oryzae* pv. *oryzae* PXO99^A. We next performed Southern blotting using *X. oryzae* pv. *oryzicola* Rs105 and *X. oryzae* pv. *oryzae* PXO99^A as subjects to verify this hypothesis experimentally. As shown in Figure 1, all three probe pairs detected bands (~7.5 kb) in the *X. oryzae* pv. *oryzae* genome, which showed the presence of the conserved gene *grx* (indicated by a solid arrow). As expected, two clear bands were detected in the *X. oryzae* pv. *oryzicola* genome using each of the three probe pairs. Of these two bands, one suggested the presence of the conserved gene *grx*, while the other (indicated by a dashed arrow) indicated the uniqueness of each tested gene (*hshA*, *hshC*, or *hshD*) in the *X. oryzae* pv. *oryzicola* Rs105 compared with *X. oryzae* pv. *oryzae* PXO99^A. In details, as shown in Figure 1, by using the probe pairs, P1-*hshA* and P2, P1-*hshC* and P2, P1-*hshD* and P2, the band corresponding to *grx* gene was detected as ~7.0, ~7.0, and ~0.6 kb in *X. oryzae* pv. *oryzicola* Rs105, respectively; the band corresponding to *hshA*, *hshC*, and *hshD* gene was determined as ~2.5, ~2.5, and ~4.5 kb in *X. oryzae* pv. *oryzicola* Rs105, respectively. The size of experimental hybridized band corresponding to each target gene was in line with our expected/predicted case in *X. oryzae* pv. *oryzicola*. The absence of *hshB* in *X. oryzae* pv. *oryzae* PXO99^A was verified experimentally in a previous study (26). Together, these findings were consistent with those of the sequence analysis presented above. Collectively, our data showed that the genome of *X. oryzae* pv. *oryzae* PXO99^A lacked homologs of the four genes (*hshA*, *hshB*, *hshC*, and *hshD*) present at the *nodB-rhgB* locus in *X. oryzae* pv. *oryzicola* Rs105. Previously, we showed that *hshB* is involved in virulence and that its transcription is regulated by DSF signaling (26). This important finding prompted us to investigate the regulation and biological functions of the three remaining genes (*hshA*, *hshC*, and *hshD*) at the *nodB-rhgB* locus of *X. oryzae* pv. *oryzicola* Rs105 (see below).

Regulation of *hshA* and *hshC* transcription by the DSF system in *X. oryzae* pv. *oryzicola*. As reported previously, the gene *hshB* within the *nodB-rhgB* locus is modulated by DSF signaling at the transcriptional level in *X. oryzae* pv. *oryzicola* (26). This finding prompted us to test whether the transcription of *hshA*,

hshC, or *hshD* is also modulated by the DSF system. For this purpose, corresponding qRT-PCR assays were performed. Using *hshB* as a positive control, we found a significant reduction in transcript abundance in a DSF-deficient mutant (Δ *rpjF*) (Fig. 2), consistent with our previous findings (26). Under the same conditions, compared with the wild-type Rs105, mutation of *rpjF* caused a significant reduced (~2.3-fold) of the transcription of *hshC*, and a dramatically reduced (~7.1-fold) of the transcription of *hshA* in *X. oryzae* pv. *oryzicola* (Fig. 2). These results suggest that the DSF system of *X. oryzae* pv. *oryzicola* regulated the transcription of *hshA* and *hshC*. To verify this point, we performed additional experiments. The DSF signal from the wild-type Rs105 was extracted, and its activity was confirmed by the DSF biosensor (Supplementary Fig. 2). Then, the validated DSF extraction was supplemented into the *rpjF* mutant to test whether the transcription of *hshA* or *hshC* could be restored to the wild-type level in *X. oryzae* pv. *oryzicola*. As expected, we found that the transcription of *hshA* or *hshC* in the *rpjF* mutant was completely restored to wild-type level by adding of exogenous wild-type DSF (Fig. 2). Meanwhile, these two genes were also partially restored to the wild-type transcriptional level when the DSF biosynthetic gene (*rpjF*) was expressed in the background of the *rpjF* mutant (Fig. 2). Collectively, these results indicate that the transcription of *hshA* or *hshC* was positively modulated by the DSF system in *X. oryzae* pv. *oryzicola*. Interestingly, disruption of the DSF system in *X. oryzae* pv. *oryzicola* did not alter the transcription of *hshD* under all tested conditions (Fig. 2).

Both *hshA* and *hshC* are required for the full virulence and in planta growth ability of *X. oryzae* pv. *oryzicola*. To examine the role of *hshA*, *hshC*, or *hshD* in the virulence of *X. oryzae* pv. *oryzicola*, we generated the corresponding in-frame deletion mutants of each gene. We then tested the virulence of the mutants by injecting bacterial cells into the rice leaves using the studied inoculated concentration (OD_{600nm} = 0.5) (18,26). Using this approach, we did not detect any visible difference in virulence between each mutant and the wild-type Rs105 (Supplementary Fig. 3A and B). As noted above, the transcription of *hshA* or *hshC* was regulated by the DSF system (Fig. 2), suggesting that the functions of these genes are linked to the cell density of *X. oryzae* pv. *oryzicola*. To test this possibility, a series of *X. oryzae* pv. *oryzicola* cell concentrations (OD_{600nm} = 0.05, 0.005, or 0.0005) was applied through injection into rice leaves for additional virulence tests. At a concentration of OD_{600nm} = 0.05 or 0.005, all three mutants (Δ *hshA*, Δ *hshC*, and Δ *hshD*) did not show visible difference in virulence in comparison with the wild-type Rs105, which was

TABLE 1. Conservation of each gene at the *nodB-rhgB* locus of *Xanthomonas oryzae* pv. *oryzicola* Rs105 in other *Xanthomonas* and related species^a

<i>X. oryzae</i> pv. <i>oryzicola</i> RS105	Description	<i>X. oryzae</i> pv. <i>oryzicola</i> BLS256 ^b	<i>X. oryzae</i> pv. <i>oryzae</i>			<i>X. axonopodis</i> pv. <i>citri</i> 306 ^b	<i>X. campestris</i> pv. <i>vesicatoria</i> 85-10 ^b	<i>X. campestris</i> pv. <i>campestris</i> ATCC33913 ^b	<i>Stenotrophomonas maltophilia</i> R551-3	<i>Xylella fastidiosa</i> 9a5c
			PXO99 ^A	MAFF311018	KACC10331					
<i>nodB</i>	Acetyltransferase	XOC_3765 (100%, 0.0)	PXO_02423 (98%, 5e-178)	XOO_0989 (98%, 1e-179)	XOO1091 (98% 2e-179)	XAC3499 (94%, 1e-170)	XCV3625 (96%, 8e-175)	XCC3367 (84%, 3e-151)	SmaL_3369 (59%, 3e-97)	XF2395 (62%, 8e-107)
<i>grx</i>	Hypothetical protein	XOC_3766 (100%, 0.0)	PXO_02424 (98%, 0.0)	XOO_0988 (98%, 0.0)	XOO_1090 (98%, 0.0)	XAC3500 (97%, 0.0)	XCV3626 (97%, 0.0)	XCC3368 (94%, 0.0)	SmaL_3370 (80%, 0.0)	XF2394 (76%, 1e-172)
–	Hypothetical protein	XOC_3767 (100%, 0.0)	N	XOO_0987 (77% 1e-27)	N	N	N	N	N	N
–	Hypothetical protein	XOC_3768 (100%, 0.0)	N	XOO_0985 (96% 2e-42)	N	N	N	N	N	N
<i>hshD</i>	Appr-1-p processing enzyme family	XOC_3769 (100%, 0.0)	N	XOO_0982 (98%, 0.0)	XOO_1084 (99%, 0.0)	N	N	N	N	N
<i>hshC</i>	Hypothetical protein	XOC_3770 (100%, 0.0)	N	N	N	XAC3502 (98%, 4e-125)	XCV3628 (96%, 1e-122)	XCC3370 (88%, 6e-106)	SmaL_3371 (56%, 9e-61)	N
<i>hshA</i>	Amidohydrolase family protein	XOC_3771 (100%, 0.0)	N	N	N	N	XCV3629 (97%, 0.0)	XCC3371 (93%, 0.0)	SmaL_3373 (76%, 0.0)	N
<i>hshB</i>	Hypothetical protein	XOC_3772 (100%, 0.0)	N	N	N	N	XCV3630 (94%, 0.0)	XCC3372 (89%, 0.0)	SmaL_3374 (73%, 0.0)	N
–	Hypothetical protein	XOC_3773 (100%, 0.0)	PXO_02429 (95%, 6e-32)	XOO_0980 (95%, 3e-33)	XOO_1079 (95%, 3e-33)	N	XCV3631 (81%, 1e-50)	XCC3376 (73%, 7e-44)	N	N
<i>rhgB</i>	Rhamnogalacturonase B	XOC_3774 (100%, 0.0)	PXO_02430 (98%, 0.0)	XOO_0978 (98%, 0.0)	XOO_1078 (98%, 0.0)	XAC3505 (87%, 0.0)	XCV3632 (86%, 0.0)	XCC3377 (80%, 2e-123)	N	N

^a Parentheses indicate percent amino acid identity (left number) and E-value (right number). N, no homologs gene was identified. –, not named.

^b Data were consistent with our previous study (26), and used here as a control.

Fig. 1. Southern blotting analysis of the uniqueness of *hshA*, *hshC*, and *hshD* in *Xanthomonas oryzae* pv. *orydicola* compared with *X. oryzae* pv. *oryzae*. **A**, The scheme of probe design and restriction sites for DNA hybridization in the present study. To test whether the homologs of *hshA*, *hshC*, or *hshD* in *X. oryzae* pv. *orydicola* are lacking in *X. oryzae* pv. *oryzae*, two probe pairs were used for each gene. Probe P1 is the internal fragment of each of the three tested genes (referred to as P1-*hshA*, P1-*hshC*, and P1-*hshD*). Each of the three fragments was used as the hybridization probe for *hshA*, *hshC*, and *hshD* of *X. oryzae* pv. *orydicola*, respectively. Probe P2 is the internal fragment of a conserved gene (*grx*, located in the *nodB-rghB* locus) in *X. oryzae* pv. *orydicola* and *X. oryzae* pv. *oryzae*. For detection of each of the three target genes, the genomic DNA of strain Rs105 (wild-type *X. oryzae* pv. *orydicola*) and strain PXO99^A (wild-type *X. oryzae* pv. *oryzae*) was digested by appreciated restriction enzymes that was shown in part A. **B**, Southern blotting verified the uniqueness of *hshA*, *hshC*, and *hshD* in the *X. oryzae* pv. *orydicola* genome compared with *X. oryzae* pv. *oryzae*. Three probe pairs, P1-*hshD* and P2, P1-*hshC* and P2, and P1-*hshA* and P2 were used to hybridize the genomic DNA of strain Rs105 (wild-type *X. oryzae* pv. *orydicola*) and strain PXO99^A (wild-type *X. oryzae* pv. *oryzae*). All three probe pairs were hybridized and detected a band (marked by solid arrows) in the *X. oryzae* pv. *oryzae* genome, which showed the presence of the conserved gene *grx*. In contrast, two clear bands were detected in the *X. oryzae* pv. *orydicola* genomes using each of the three probe pairs (part A). Of these two bands, the one marked by solid arrows suggests the presence of the conserved gene *grx*; the other (marked by the dashed arrow) indicates the uniqueness of each tested gene (*hshA*, *hshC*, or *hshD*) in the *X. oryzae* pv. *orydicola* genome.

Fig. 2. Differential regulation of the transcription of *hshA*, *hshC*, and *hshD* in *Xanthomonas oryzae* pv. *orydicola* by the diffusible signal factor (DSF) system. Disruption of the DSF system led to about a twofold decrease (highlighted with a white dash line) in the transcription of *hshA* or *hshC* in *X. oryzae* pv. *orydicola*. Each column indicates the mean of three biologically independent experiments. Vertical bars represent standard errors. * ($P < 0.05$; *t* test) above the bars indicate a significant difference of transcriptional level of *hshA* or *hshC* in the DSF-deficient strain ($\Delta rpfF$) compared with that in the wild-type strain Rs105. $\Delta rpfF$ (*rpfF*), the *rpfF* complemented strain; $\Delta rpfF$ +DSF, the cells of the *rpfF* mutant were treated by the DSF extraction of the wild-type Rs105. The transcription of *hshB* in $\Delta rpfF$ was used as a positive control and found to be consistent with our previous results (26).

evaluated based on the water-soaking lesion length. Surprisingly, two mutants ($\Delta hshA$ and $\Delta hshC$) exhibited a significant reduction (~ 2.0 -fold for $\Delta hshA$; ~ 2.1 -fold for $\Delta hshC$) in virulence when using the lowest ($OD_{600nm} = 0.0005$) of the tested concentrations, whereas their corresponding complemented strains retained wild-type ability in this function. Using the same method, we also tested the virulence of the double mutant ($\Delta hshCA$) (Supplementary Table 3), and found that in comparison with the wild-type Rs105, this double mutant only exhibited a significant reduction (~ 2.3 -fold) in virulence when using the low ($OD_{600nm} = 0.0005$), but not the relative high (0.5, 0.05, or 0.005) tested concentrations (Supplementary Fig. 4). Interestingly, at all cell concentrations used, the $hshD$ deletion mutant did not exhibit altered virulence, indicating that this gene was not involved in the virulence of *X. oryzae* pv. *oryzicola*. Notably, in all experiments, the $hshB$ mutant showed a consistent and remarkable decrease in virulence, in accordance with our previous findings (26). From the results of our DNA hybridization and pathogenicity tests (Figs. 1 and 3A), we concluded that $hshA$ and $hshC$, two genes in the *nodB-rhgB* locus of *X. oryzae* pv. *oryzicola* Rs105 that were missing from *X. oryzae* pv. *oryzae* PXO99^A, were involved in *X. oryzae* pv. *oryzicola* virulence. Furthermore, these two genes ($hshA$ and $hshC$) probably did not

have a cumulative contribution to the virulence of *X. oryzae* pv. *oryzicola* Rs105.

Next, to explore whether the involvement of $hshA$ or $hshC$ in virulence is related with the in planta colonization of the corresponding mutant, we detected the cell populations of these two mutants in the infected rice leaves. For this purpose, we used a low concentration ($OD_{600nm} = 0.0005$) of the wild-type Rs105 and each mutant to inoculate rice leaves because in this way the role of the two genes in virulence could be visualized (Fig. 3A). We then recovered the bacterial cells from the infected rice leaves 10 days after inoculation. As shown in Figure 3B, the two mutants ($\Delta hshA$ and $\Delta hshC$) exhibited a remarkable decrease in cell numbers in comparison with the wild-type strain when recovered after 10 days. Under the same conditions, the corresponding complemented strain of $hshA$ or $hshC$ mutant exhibited wild-type level in this function, and the $hshB$ mutant showed a significant decrease in cell populations, consistent with our previous findings (26). Taken together, these results suggested that mutation of $hshA$ and $hshC$ reduced the in planta growth ability of *X. oryzae* pv. *oryzicola*, which was one of the probable mechanisms underlying the involvement of these two genes in virulence.

Fig. 3. Mutation of $hshA$ and $hshC$ reduced the virulence level and bacterial load on host rice using the low *Xanthomonas oryzae* pv. *oryzicola* inoculated cell density. **A**, Statistical analysis of the virulence of *X. oryzae* pv. *oryzicola* strains on host rice using a low ($OD_{600nm} = 0.0005$) inoculated cell density. **B**, In planta colonization ability of various *X. oryzae* pv. *oryzicola* strains in susceptible rice leaves. Bacteria were recovered from infected rice leaves (Shanyou-63) 10 days after inoculation using the low ($OD_{600nm} = 0.0005$) inoculated cell density. Each column indicates the mean of three biologically independent experiments. Vertical bars represent standard errors. * ($P < 0.05$; t test) above the bars indicate a significant difference between mutant and the wild-type Rs105 in tested phenotypes. Rs105, the wild-type strain of *X. oryzae* pv. *oryzicola*; $\Delta hshD$, the $hshD$ deletion mutant; $\Delta hshC$, the $hshC$ deletion mutant; $\Delta hshA$, the $hshA$ deletion mutant; $\Delta hshC$ ($hshC$), the complemented strain of $\Delta hshC$; $\Delta hshA$ ($hshA$), the complemented strain of $\Delta hshA$; and $\Delta hshB$, the $hshB$ deletion mutant, were used as a positive control (26).

Both *hshA* and *hshC* are required for the epiphytic survival level of *X. oryzae* pv. *oryzicola*. Both *hshA* and *hshC* required for the full virulence in low density would lead to question whether they play an important role in the initial stages of inoculation. To test this point, we performed additional experiments to test whether the epiphytic survival in the early colonization stage is affected when *hshA* or *hshC* was mutated. As shown in Figure 4A, the *hshA* and *hshC* mutant exhibited a reduction on the cell populations recovered from the rice leaf surface at all the selected time points (0.5, 4, 12, 20, and 28 h) in comparison with the wild-type Rs105. Meanwhile, the complemented strain of *hshA* or *hshC* mutant restored to wild-type level in recovered cell populations. By using an AUPDC methodology, we found that the *hshA* or *hshC* mutant showed a significant reduction ($P < 0.05$; t test) of epiphytic survival (cell numbers) in comparison with the wild-type strain during the total of 28-h colonization process (Fig. 4B). Furthermore, the complemented strain of *hshA* and *hshC* mutant retained wild-type level in this function. Collectively, these results suggest that *hshA* and *hshC* were probably involved in the epiphytic fitness of *X. oryzae* pv. *oryzicola*, which also probably contributed to the reduced virulence of the *hshA* and *hshC* mutant.

The involvement of *hshA* and *hshC* in virulence is not associated with the several characterized virulence-related traits in *X. oryzae* pv. *oryzicola*. Previously, we demonstrated the involvement of *hshB* in virulence, probably through impairing several important virulence-related traits, including EPS production and antioxidative activity (26). To test whether *hshA* and

hshC could affect virulence-related traits and, in turn, modulate virulence, we compared the wild-type Rs105 and the $\Delta hshA$, $\Delta hshC$ or double mutant in terms of four well-studied virulence-associated functions, including in vitro growth, extracellular protease activity, resistance to oxidative stress, and EPS production. Unexpectedly, no visible alteration in the tested phenotypes/functions was detected between each single mutant or double mutant and the wild-type strain (Supplementary Figs. 5 to 9). These results indicate that the involvement of *hshA* and *hshC* in virulence was independent of our tested virulence-associated functions.

DISCUSSION

The present study indicates that the *nodB-rhgB* locus in *X. oryzae* pv. *oryzicola* Rs105 affected virulence. This locus displayed two interesting features in terms of its sequence and genomic organization. One is that the three genes (*nodB*, *grx*, and *rhgB*) at this locus were highly conserved in different *Xanthomonas* and related species (Table 1). However, their functions have not been described. The other is that the seven remaining genes exhibited diversity in terms of number; different *Xanthomonas* species utilizing different infection mechanisms may possess any number of homologs of these seven genes in *X. oryzae* pv. *oryzicola*. For instance, similar to *X. oryzae* pv. *oryzicola* Rs105, *X. axonopodis* pv. *citri* 306, and *X. campestris* pv. *vesicatoria* 85-10 are all mesophilic pathogens; three virulence-associated genes (*hshA*, *hshB*, and *hshC*) were all present in the *X. campestris* pv. *vesicatoria* 85-10 genome,

Fig. 4. Mutation of *hshA* and *hshC* reduced the epiphytic survival level of *Xanthomonas oryzae* pv. *oryzicola*. **A**, Time-course determination of epiphytic survival level of various *X. oryzae* pv. *oryzicola* strains. Bacteria were recovered from rice leaf surfaces at various times (0.5, 4, 12, 20, and 28 h) after spraying. **B**, The area under population dynamics curve (AUPDC)-based calculation of epiphytic survival of various *X. oryzae* pv. *oryzicola* strains. Rs105, the wild-type strain of *X. oryzae* pv. *oryzicola*; $\Delta hshD$, the *hshD* deletion mutant; $\Delta hshC$, the *hshC* deletion mutant; $\Delta hshA$, the *hshA* deletion mutant; $\Delta hshC$ (*hshC*), the complemented strain of $\Delta hshC$; and $\Delta hshA$ (*hshA*), the complemented strain of $\Delta hshA$. Three replicates were used for each treatment, and the experiment was performed three times. Vertical bars represent standard errors. * ($P < 0.05$; t test) above the bars indicate a significant difference between the wild-type strain and tested mutants.

but only one (*hshC*) was detected in the *X. axonopodis* pv. *citri* 306 genome. Interestingly, although *X. campestris* pv. *campestris* ATCC33913 is a vascular pathogen, it contains all of the homologs of these three virulence-related genes from *X. oryzae* pv. *oryzicola* Rs105. More interestingly, three model *X. oryzae* pv. *oryzae* strains, *X. oryzae* pv. *oryzae* PXO99^A, *X. oryzae* pv. *oryzae* MAFF311018, and *X. oryzae* pv. *oryzae* KACC10331 lacked all of these three genes, despite the fact that *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* are highly similar in taxonomy and genome structure. Currently, the role of this locus in the above-noted *Xanthomonas* species has not been fully described. Elucidation of its role and mechanisms will provide useful information for understanding whether this locus or certain genes within this locus plays a conserved role in different *Xanthomonas* species.

Previously, we showed that *hshA* and *hshB* are cotranscribed (26). The gene *hshB* was previously shown to be controlled both by the DSF system (26). In the present study, we further showed that the patterns of transcriptional regulation of *hshA* and *hshB* by the DSF system are highly consistent; this finding was in accordance with our qRT-PCR results. Interestingly, although *hshA* and *hshB* are cotranscribed and share similar domains, they exhibit different functions. They were both involved in the bacterial colonization of rice tissues (Fig. 3B); however, *hshB* plays a critical and distinct role in extracellular protease activity, oxidative stress, and EPS production in *X. oryzae* pv. *oryzicola*; these functions were not found to be regulated by *hshA* (Supplementary Figs. 6 to 8). Our results indicate that the two cotranscribed and similar domain-containing proteins (HshA and HshB) at this locus contributed to the virulence of *X. oryzae* pv. *oryzicola* via shared and distinct mechanisms. In addition to *hshA*, another interesting virulence-associated gene within this locus is *hshC*. In the *X. oryzae* pv. *oryzicola* genome, the predicted transcriptional direction of this gene was opposite to that of *hshA*, indicating that this gene might not be cotranscribed with *hshA*. Although *hshC* is annotated as a hypothetical protein of unknown function in *X. oryzae* pv. *oryzicola*, we however, found it was involved in the virulence and regulated by the DSF system in *X. oryzae* pv. *oryzicola*. Interestingly, although *hshA* and *hshC* were both involved in virulence, they, however, did not exhibit an accumulative effect on virulence. Addressing the precise molecular virulence mechanism used by *hshA* and *hshC* is deserved for further studies.

The DSF signaling pathway is a conserved system in *Xanthomonas* (9,20). This system regulates virulence and virulence-associated traits in diverse *Xanthomonas* species; however, the regulatory mechanism is different in some species. For example, the DSF system positively regulates motility and negatively modulates biofilm formation in *X. campestris* pv. *campestris*, but this mechanism is different in *X. oryzae* pv. *oryzae* (20). In this study and in our previous report, we found that three genes (*hshA*, *hshB*, and *hshC*) within the *nodB-rhgB* locus were controlled by DSFs and that they were involved in virulence (Figs. 2 and 3) (26). Interestingly, homologs of these three genes were absent in *X. oryzae* pv. *oryzae*. This finding suggests that *X. oryzae* pv. *oryzicola* utilized a DSF-controlled and *X. oryzae* pv. *oryzae*-independent means of regulating virulence. A systematic analysis of the effect of these three genes on gene/protein expression at the genome-wide level both in *X. oryzae* pv. *oryzicola* (wild-type versus mutant strains) and *X. oryzae* pv. *oryzae* (wild-type versus over-expression strains) will help us understand how they modulate virulence in *X. oryzae*. Although *X. oryzae* pv. *oryzae* lacks homologs of these three genes, *X. campestris* pv. *campestris* possesses them. This suggests that the homologs of these three genes are also controlled by DSF signaling and influence the virulence of *X. campestris* pv. *campestris*. However, a previous microarray study showed that not all of them were regulated at the transcriptional level by DSF signaling (12). This finding indicates that the transcriptional regulation of these three genes by the DSF system varies among *Xanthomonas* species. We currently do not know their functions in the virulence of

X. campestris pv. *campestris*; further elucidation of this function in *X. campestris* pv. *campestris* will expand our understanding of the DSF regulatory mechanisms in different species of *Xanthomonas*.

In summary, this study describes two novel DSF-controlled, virulence-related genes in the *nodB-rhgB* locus of *X. oryzae* pv. *oryzicola*. These two genes (*hshA* and *hshC*) were experimentally found to be unique in *X. oryzae* pv. *oryzicola* Rs105 compared with *X. oryzae* pv. *oryzae* PXO99^A. The decrease of *hshA* or *hshC* in virulence was associated with the reduced in planta growth and epiphytic survival level of the *hshA* or *hshC* mutant in *X. oryzae* pv. *oryzicola* Rs105.

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