epv*, Encoding a Hypothetical Protein, Is Regulated by DSF-Mediating Quorum Sensing as Well as Global Regulator Clp and Is Required for Optimal Virulence in *Xanthomonas oryzae* pv. *oryzicola

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ABSTRACT

Qian, G., Zhang, Y., Zhou, Y., Liu, C., Zhao, Y., Song, Z., Fan, J., Hu, B., and Liu, F. 2012. *epv*, encoding a hypothetical protein, is regulated by DSF-mediated quorum sensing as well as global regulator Clp and is required for optimal virulence in *Xanthomonas oryzae* pv. *oryzicola*. *Phytopathology* 102:841-847.

Xanthomonas oryzae pv. *oryzicola* causes bacterial leaf streak in rice, a destructive disease worldwide. In this study, six putative hypothetical secreted proteins, which were absent in *X. oryzae* pv. *oryzae*, were detected from *X. oryzae* pv. *oryzicola* strain BLS256. Disruption-based mutagenesis study revealed that one of them, *Xoc_15235*, named as extracellular polysaccharide and virulence-related gene (*epv*), was required for the optimal virulence in host rice but not for the induction of a hypersensitive reaction in nonhost tobacco. Sequence analysis revealed that *epv* was highly conserved in *Xanthomonas* spp. (except *X. oryzae* pv.

oryzae). In-frame deletion of *epv* in *X. oryzae* pv. *oryzicola* dramatically impaired pathogen virulence and extracellular polysaccharide (EPS) production, one of the important known virulence-associated functions in *Xanthomonas* spp. Quantitative real-time reverse-transcription polymerase chain reaction showed that expression of both *gumB* (a gene encoding exopolysaccharide xanthan biosynthesis export protein) and a known virulence-related gene, *pgk* (encoding phosphoglycerate kinase), were obviously reduced in the *epv*-deletion mutant compared with the wild-type strain Rs105. In addition, we observed that *epv* was positively regulated by both diffusible signal factor and global regulator Clp in *X. oryzae* pv. *oryzicola*. Taken together, the novel roles and genetics of *epv* of *X. oryzae* pv. *oryzicola* in the EPS production and virulence were investigated for the first time.

Additional keyword: pathogenicity.

The genus *Xanthomonas* consists of mainly phytopathogenic bacteria, which infect a wide variety of economically important plants (28). Among them, *Xanthomonas oryzae* pv. *oryzicola*, the causal agent of bacterial leaf streak (BLS) of rice (*Oryza sativa*), constrains production of this staple crop in much of Asia and parts of Africa (19). *X. oryzae* pv. *oryzicola* enters rice leaves mainly through leaf stomata or wounds, multiplies in the substomatal cavity, colonizes the parenchyma apoplast, and causes water-soaked interveinal lesions that develop into translucent streaks (19,27,32). To date, the virulence mechanisms of *X. oryzae* pv. *oryzicola* are not clearly known, and efficient control measures for BLS are lacking.

Secreted proteins of pathogenic or symbiotic bacteria form important interactions with their host in the host extracellular environment (20). These proteins are demonstrated to be involved in various functions, including provision of nutrients, cell-to-cell communication, and detoxification of the environment (24). Furthermore, secreted proteins of intracellular pathogens are known to be the primary antigen targets for host immune response (13). Thus, these proteins may be the key factors to induce immune protection, and could be regarded as the potential sources of vaccine development (25).

In plant-associated bacteria, extracellular proteins are those proteins secreted into culture or delivered into plant cells when

they were grown in medium or infection in plants by relying on several protein secretion systems, including type I, II, III, and VI secretion system (T1SS, T2SS, T3SS, and T6SS, respectively) or the twin-arginine translocation pathway (2). Among them, the T3SS encoded by the hypersensitive reaction and pathogenicity (*hrp*) genes cluster is responsible for its effectors' translocation into plant cells to trigger disease symptoms in susceptible host or the hypersensitive reaction (HR), a programmed cell death in nonhosts restricted to the area of infection (28). Another group of extracellular proteins are mostly T2SS-dependent degradative enzymes (28). They are actively secreted and supported during the early and late stages of infection, and most of them are virulence factors (28). These findings suggested that bacterial extracellular proteins play critical roles in bacterium-plant interaction.

A hypothetical protein is a protein whose existence has been predicted but for which there is no experimental evidence that it is expressed in vivo. Bioinformatic analysis showed that, in the published genome of *Xanthomonas*, a number of open reading frames (ORFs) were predicted as hypothetical proteins. Recently, several hypothetical proteins were demonstrated to be involved in bacterial pathogenicity in different *Xanthomonas* spp., such as *XALc_2347*, *XALc_22682*, *XALc_0742*, and *XALc_0885* in xylem-invading pathogen *X. albilineans* (21); and *Xoryp_02235*, *Xoryp_00885*, and *Xoryp_22910* in *X. oryzae* pv. *oryzicola* (9). However, the roles of most hypothetical proteins in bacterial virulence are largely unknown in *Xanthomonas* spp., including *X. oryzae* pv. *oryzicola*.

In a previous study, we reported that HshB protein (*Xoryp_18565*) was required for virulence and regulated by both diffusible signal factor (DSF)-mediating quorum sensing (QS) and global regulator Clp in *X. oryzae* pv. *oryzicola* (29). Inter-

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estingly, in the genome of *X. oryzae* pv. *oryzicola* strain BLS256 (<http://cmr.jcvi.org/cgi-bin/CMR/GenomePage.cgi?org=Xoc>), this protein is annotated as the hypothetical secreted protein with hydrolase activity, although blastp search indicated that this protein was a homolog of imidazolonepropionase (29). More interestingly, no homologue of this protein is detected in the whole genome of *X. oryzae* pv. *oryzae*, which is a xylem-invading pathogen (colonies in the vascular tissues and propagates in the xylem of rice leaves) and causes bacterial blight of rice (4). These results showed the novel roles of unique putative hypothetical secreted protein in *X. oryzae* pv. *oryzicola* compared with *X. oryzae* pv. *oryzae*. Based on this finding, we made a survey in the genome of *X. oryzae* pv. *oryzicola* strain BLS256 (NZ_AAQ01000001), and found another six hypothetical secreted proteins (Xoryp_4540, Xoryp_11980, Xoryp_15235, Xoryp_18640, Xoryp_20851, and Xoryp_22495), which were predicted to be absent in the genome of *X. oryzae* pv. *oryzae* (<http://www.ncbi.nlm.nih.gov/genomes/blast.cgi?taxid=347>) by comparative genomics (Supplemental Tables 1 and 2).

Thus, the objective of this work was to understand the individual role of these six hypothetical secreted proteins in *X. oryzae* pv. *oryzicola*. In the present work, a mutagenesis study revealed that one of them, *Xoc_15235*, was required for the optimal virulence. Then, the genetics and biological roles of *Xoc_15235* in the virulence and virulence-associated functions were presented. Finally, according to our experimental results, we defined *Xoc_15235* as the extracellular polysaccharide and virulence-related (*epv*) gene in *X. oryzae* pv. *oryzicola*.

MATERIALS AND METHODS

Strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this work were listed in Table 1. *Escherichia coli* strains were cultivated at 37°C in Luria-Bertani (LB) medium or on LB agar plates. Unless specified otherwise,

X. oryzae pv. *oryzicola* and *X. oryzae* pv. *oryzae* 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). When required, antibiotics were added to the medium as the following final concentrations: kanamycin (Km), 50 µg/ml; rifampicin (Rif), 100 µg/ml; and gentamicin (Gm), 20 µg/ml.

Generation of independent disruption mutants of six genes encoding for hypothetical secreted proteins in *X. oryzae* pv. *oryzicola*. By analyzing the genome of *X. oryzae* pv. *oryzicola* strain BLS256, six hypothetical secreted proteins, including Xoryp_4540, Xoryp_11980, Xoryp_15235 (named as *Epv*), Xoryp_18640, Xoryp_20851, and Xoryp_22497, were detected. To investigate the role of each gene, these six genes were disrupted individually by using single cross-over integration protocol (4). In brief, the internal DNA fragment of each target gene (Supplemental Table 3) was amplified from the genome of wild-type strain Rs105 by using different primer pairs. The targeted internal DNA fragment of each gene was digested with appreciated enzymes, and cloned into the corresponding sites of suicide vector pK18mobsacB (31), resulting in six correct recombinant plasmids. Then, each recombinant plasmid was introduced into *X. oryzae* pv. *oryzicola* wild-type strain Rs105 by electroporation. The transconjugants were selected on NA plates without sucrose and with Rif (100 µg/ml) and Km (50 µg/ml). The resultant disruption mutant of each gene was confirmed by polymerase chain reaction (PCR) amplification (Supplemental Table 4).

Generation of *epv*-deletion mutant. The *X. oryzae* pv. *oryzicola* wild-type strain Rs105 was used as a parental strain to generate the in-frame deletion mutant via double cross-over homologous recombination. The in-frame deletion of *epv* was performed as described previously (29,30). In brief, two *epv* flanking regions were generated by PCR using primer pairs: *epv*-1F/*epv*-1R and *epv*-2F/*epv*-2R, respectively. The fragment *epv*-F

TABLE 1. Strains and plasmids used in this study^a

Strains and plasmids	Characteristics	Source
<i>Escherichia coli</i>		
DH5α	Φ80 <i>lacZ</i> Δ <i>M15</i> , Δ(<i>lacZYA-argF</i>) <i>U169</i> . <i>recA1</i>	Lab collection
<i>Xanthomonas oryzae</i> pv. <i>oryzicola</i>		
Rs105	Rif ^R , Wild-type strain	Lab collection
Δ <i>rpfF</i>	Rif ^R , Deletion of <i>rpfF</i> in wild-type Rs105	30
Δ <i>clp</i>	Rif ^R , Deletion of <i>clp</i> in wild-type Rs105	29
Δ <i>hshB</i>	Rif ^R , Deletion of <i>hshB</i> in wild-type Rs105	29
Δ <i>Xoryp_4540</i>	Rif ^R , Km ^R , disruption of <i>Xoc_4540</i> in wild-type Rs105	This study
Δ <i>XorypC_11989</i>	Rif ^R , Km ^R , disruption of <i>Xoc_11989</i> in wild-type Rs105	This study
Δ <i>epv-D</i>	Rif ^R , Km ^R , disruption of <i>Xoc_15235</i> in wild-type Rs105	This study
Δ <i>Xoryp_18640</i>	Rif ^R , Km ^R , disruption of <i>Xoc_18640</i> in wild-type Rs105	This study
Δ <i>Xoryp_20851</i>	Rif ^R , Km ^R , disruption of <i>Xoc_20851</i> in wild-type Rs105	This study
Δ <i>Xoryp_22497</i>	Rif ^R , Km ^R , disruption of <i>Xoc_22497</i> in wild-type Rs105	This study
Δ <i>Xoryp_15235</i>	Rif ^R , Km ^R , Deletion of Δ <i>XOC_15235</i> in wild-type Rs105	This study
Δ <i>epv</i>	Rif ^R , Deletion of Δ <i>XOC_15235</i> in wild-type Rs105	This study
Δ <i>epv (epv)</i>	Rif ^R , Gm ^R , Δ <i>XOC_15235</i> contains plasmid pB15235	
<i>X. oryzae</i> pv. <i>oryzae</i>		
PXO99	Wild-type strain	Lab collection
PXO99 (pBEPV)	PXO99 contains plasmid pBEPV	This study
Plasmids		
pK18mobsacB	Km ^R , Allelic exchange suicide vector, <i>sacB</i> ori(T(RP4))	31
pBBR1-MCS5	Broad-host-range vector with a P _{lac} promoter	14
pK184540	Km ^R , pK18mobsacB contains a 787-bp internal DNA fragment of <i>Xoc_4540</i>	This study
pK1811989	Km ^R , pK18mobsacB contains a 462-bp internal DNA fragment of <i>Xoc_11989</i>	This study
pK1815235	Km ^R , pK18mobsacB contains a 541-bp internal DNA fragment of <i>Xoc_15235</i>	This study
pK1818640	Km ^R , pK18mobsacB contains a 554-bp internal DNA fragment of <i>Xoc_18640</i>	This study
pK1820851	Km ^R , pK18mobsacB contains a 554-bp internal DNA fragment of <i>Xoc_20851</i>	This study
pK1822497	Km ^R , pK18mobsacB contains a 482-bp internal DNA fragment of <i>Xoc_22497</i>	This study
pK18FR	Km ^R , pK18mobsacB contained two <i>Xoc</i> -15235 flanking fragments (430- and 400-bp length, respectively)	This study
pBEPV	Gm ^R , pBBR1-MCS5 cloned with a 920-bp DNA fragment containing intact <i>epv</i> gene and its predicted promoter	This study

^a Rif^R, Km^R, and Gm^R indicated resistance to rifampicin, kanamycin, and gentamicin, respectively; *epv* = extracellular polysaccharide and virulence-related gene.

(439-bp length, digested with *Bam*HI and *Kpn*I) and the fragment *epv*-R (400-bp length, digested with *Kpn*I and *Xba*I) were ligated into pK18mobsacB digested with *Bam*HI and *Xba*I. This recombinant vector, designated as pK18FR, was transformed into the wild-type strain Rs105 by electroporation. 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 *epv* in-frame deletion was confirmed by PCR and Southern blotting (data not shown). One of the confirmed mutants, named Δepv , was selected for further study.

Complementation of the *epv* mutant. The mutation of *epv* was complemented as described previously (29,30). In brief, a 920-bp DNA fragment containing the intact *epv* and its predicted promoter region was amplified from the wild-type strain Rs105 with primers *epv*-CF (5'-CGGAATTCATCAACGACGGCAAGCGGT-3', *Eco*RI site underlined) and *epv*-CR (5'-CGGGA^TTCCAACAGCGATGGAAGAACGAC-3', *Bam*HI site underlined). The amplified fragment was digested with enzyme *Bam*HI/*Eco*RI, and cloned into *Bam*HI/*Eco*RI-digested pBBR1-MCS5, resulting in complemented plasmid pBEPV (Table 1). Plasmid pBEPV was transformed into the competent cell of Δepv by electroporation. Finally, one positive complemented strain, named Δepv (*epv*) was selected on NA plates with Rif (100 µg/ml) and Gm (50 µg/ml) for further study.

Pathogenicity testing. Pathogenicity assays were performed in a glasshouse at 22 to 30°C as previously described (29,30). In brief, *X. oryzae* pv. *oryzae* strains were cultivated in NB medium at 28°C with appropriate antibiotics. The strains were adjusted to an optical density at 600 nm of ≈ 0.1 , then inoculated into leaves of 2-month-old rice plants ('Shanyou-63', susceptible to the pathogen) by using leaf needling, or infiltrated into leaves of 2-week-old rice seedlings (Shanyou-63, susceptible to the pathogen) by needleless syringe. Lesion lengths on adult rice leaves were measured after 14 days of inoculation. Ten leaves were inoculated for each *X. oryzae* pv. *oryzicola* strain in each treatment. The same experiment was repeated three times.

Quantification of extracellular polysaccharide production. Extracellular polysaccharide (EPS) production of the bacterium was measured with some modification, as described previously (23,30). Briefly, *X. oryzae* pv. *oryzicola* or *X. oryzae* pv. *oryzae* 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 supernate with ethanol, then 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.

Real-time PCR. Relative quantitative real-time PCR was carried out using the SYBR Premix EX Tag 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 are listed in Table 2. RNA was extracted from *X. oryzae* pv. *oryzicola* wild-type strain Rs105, and Rs105-derivative mutants ($\Delta rpfF$, Δclp , $\Delta hshB$, and Δepv) (Table 1) by 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 µg of each RNA sample was used to synthesize cDNA with a cDNA synthesis kit (TaKaRa). The same experiment was repeated three times. In this study, the transcriptional levels of *epv* in the strains of the wild-type strain, $\Delta rpfF$ (DSF-deficient mutant) (30), and Δclp (29) were assessed and compared, respectively. The transcriptional levels of *wxolB* (*Xoryp*_3864), *pgk* (*Xoryp*_3589), *gapA* (*Xoryp*_3592), *tal-C10c-like* (*Xoryp*_4384), *dsbC* (*Xoryp*_3811), *rfaA* (*Xoryp*_3845), *atmR* (*Xoryp*_2825), *thrC* (*Xoryp*_2208), *purF* (*Xoryp*_1105), *trpA* (*Xoryp*_2950), and *gumB* (*Xoryp*_1867) in the wild-type strain and the *epv*-deletion mutant was assessed and compared.

Data analysis. All statistical analyses were conducted using Statistical Package for the Social Science (SPSS) 14.0 (SPSS Inc., Chicago). The analysis of variance procedure of SPSS and Fisher's least significant difference test or hypothesis test of percentages (*t* test), *P* = 0.05, were used to determine significant differences in pathogenicity tests, EPS determination and gene expressions.

RESULTS

Gene *epv* of *X. oryzae* pv. *oryzicola* was required for pathogen optimal virulence in host rice but not for triggering hypersensitive reaction in nonhost tobacco. To investigate the individual role of six putative hypothetical secreted proteins in *X. oryzae* pv. *oryzicola*, the corresponding disruption mutant of each gene was generated and confirmed (Table 1; Supplemental Figure S1). The results of pathogenicity tests showed that only one of these six hypothetical secreted proteins, *Epv* (*Xoryp*_15235), was required for the optimal virulence on rice. Disruption of *epv* impaired pathogen virulence on both adult rice plants and rice seedlings, and five other hypothetical secreted protein-defective mutants showed similar wild-type ability in virulence on host rice (Supplemental Figure S2). To confirm the contribution of *epv* to *X. oryzae* pv. *oryzicola* virulence, the *epv*-deletion mutant (Δepv) was constructed and the corresponding virulence test was performed again. We observed that the *epv*-deletion mutant displayed a significant reduction of virulence on both adult rice plants and rice seedlings (Fig. 1), whereas the corresponding complemented strain showed wild-type virulence ability (Fig. 1). The results of pathogenicity tests were consistent with that of the *epv*-disruption mutant. Together, these results revealed that *epv* was required for *X. oryzae* pv. *oryzicola* virulence on host rice.

TABLE 2. Primers for quantitative real-time polymerase chain reaction

Gene	Primer sequence for 5' sequence	Primer sequence for 3' sequence	Primer source	Gene source
16S rRNA	AATGGGCGCAAGCCTGATC	AACCACCACCTACGCACGC	29	29
<i>epv</i> (<i>Xoryp</i> _15235)	CGGCAACACGATCTACTTCA	ATTCCAGATTACGGTGAGC	This study	This study
<i>gumB</i> (<i>Xoryp</i> _1867)	AACTGATCGGACGATTTTGC	GCTTGGAGACCAGTTCTTCG	This study	This study
<i>tal-C10c-like</i> (<i>Xoryp</i> _4384)	ACCGTGATGTGGGAACAAGATG	GAGGCAATAGCTCCCTCAACC	This study	9
<i>dsbC</i> (<i>Xoryp</i> _3811)	GCATTCAAGGATTTCGGTGCG	ATGGTGACGGGGCGGTCTTC	This study	9
<i>wxocB</i> (<i>Xoryp</i> _3864)	CCGCGTACTGGACGATAAGT	TGCGATAAGAGCGTTGACAC	This study	27
<i>pgk</i> (<i>Xoryp</i> _3589)	CGCAGTGGACAAGTACGACA	CTTGCCCTCCAGAACTCCA	This study	27
<i>gapA</i> (<i>Xoryp</i> _3592)	GCTGGGGTATACCGAAGACA	TCGTTGTGCTACCAAGGACAC	This study	27
<i>rfaA</i> (<i>Xoryp</i> _3845)	GGCGTTGTGTTCAATCCTTGT	AGTCGCGACCGATCAAATAC	This study	9
<i>atmR</i> (<i>Xoryp</i> _2825)	GTCGGTGGGCAGGTGCTGTA	GACGATGCGGTGCGCTTT	This study	9
<i>thrC</i> (<i>Xoryp</i> _2208)	ATGCGTTGGCGTTGATTCTG	CGATGGCCTTCTCCACCCT	This study	9
<i>purF</i> (<i>Xoryp</i> _1105)	CAGGATCTGGAAGACCTGGA	TGATGTAATCGCCGTTGAAG	This study	9
<i>trpA</i> (<i>Xoryp</i> _2950)	CTTGCGTACGTGTTGGAAGC	TCTCGATCGGGTTGAGGTAG	This study	9

HR, a programmed cell death, is a plant's mechanism to prevent the spread of infection by microbial pathogens. It serves to restrict the growth and spread of pathogens to other part of the plant. To understand the individual role of six hypothetical proteins of *X. oryzae* pv. *oryzicola* in triggering HR in nonhost tobacco, the corresponding experiment was performed. All six hypothetical protein-disruption mutants, including Δepv -D, were able to trigger HR in nonhost tobacco (Supplemental Figure S3). Furthermore, the *epv*-deletion mutant Δepv was also able to trigger HR in nonhost tobacco (data not shown). These results indicated that *epv* was not required for triggering HR in nonhost tobacco.

Mutation of *epv* in *X. oryzae* pv. *oryzicola* impaired pathogen ability in EPS production. EPS production is regarded as an important virulence-associated function in *Xanthomonas* spp. To understand whether or not deletion of *epv* would impair the pathogen ability in EPS production, the corresponding assay was performed. It was evident that the *epv* mutant exhibited a significant decrease in EPS production compared with the wild-type strain (Fig. 2A). To further confirm this result, the transcriptional level of *gumB*, a gene encoding exopolysaccharide xanthan biosynthesis export protein, was assessed in the wild-type strain and the *epv*-deletion mutant. We observed that the transcriptional expression level of *gumB* was significantly decreased in the *epv*-deletion mutant compared with the wild-type strain Rs105 (Fig. 2B). Taken together, these results indicated that *epv* was involved in the EPS production in *X. oryzae* pv. *oryzicola*.

Fig. 1. Pathogenicity test of *Xanthomonas oryzae* pv. *oryzicola* strains in rice. **A**, Lesion lengths on the leaves of adult susceptible rice plants ('Shanyou63', 2 months old) inoculated with *X. oryzae* pv. *oryzicola* strains. **B**, Lesion lengths on the leaves of rice seedlings ('Shanyou63', 2 weeks old) by infiltration with *X. oryzae* pv. *oryzicola* strains. **C**, Calculated data of lesion length on the leaves of adult susceptible rice ('Shanyou63', 2 months old) inoculated with *X. oryzae* pv. *oryzicola* strains. Rs105, wild-type strain of *X. oryzae* pv. *oryzicola*; Δepv , extracellular polysaccharide and virulence-related (*epv*) gene deletion mutant of *X. oryzae* pv. *oryzicola*; Δepv (epv), complemented strain of Δepv . Vertical bars represent standard errors of the means. Different letters above the data bars indicate a significant difference between the wild-type strain and *epv*-deletion mutant or *epv* complemented strain ($P < 0.05$, *t* test).

Transcriptional expression of *epv* was positively regulated by DSF-mediated QS and global regulator Clp. To understand whether or not *epv* was regulated by DSF-mediated QS and global regulator Clp, the transcriptional levels of *epv* in the wild-type strain Rs105, QS-deficient strain $\Delta rpfF$, and *clp* mutant Δclp were measured and compared. It was observed that the transcriptional level of *epv* in the DSF-deficient strain $\Delta rpfF$ was reduced compared with wild-type strain Rs105, indicating that *epv* was regulated by DSF-mediated QS (Fig. 3). Furthermore, it was also shown that the transcriptional level of *epv* was reduced in the *clp* mutant (Fig. 3), indicating that *epv* was also positively regulated by global regulator Clp. Thus, these results indicated that *epv* was positively regulated by both DSF-mediated QS and Clp.

Gene *epv* was not involved in growth, resistance to oxidative stress, or protease activity in *X. oryzae* pv. *oryzicola*. Growth, resistance to oxidative stress, and protease activity were demonstrated to be the important virulence-associated functions in *Xanthomonas* spp. (4,11,23,29). Because the mutation of *epv* in *X. oryzae* pv. *oryzicola* impaired pathogen virulence on host rice, it is reasonable to investigate whether or not *epv* mutation affected virulence-associated functions described above, resulting in its virulence decrease on rice. Unexpectedly, in this study, we observed that *epv* mutation did not impair the ability of *X. oryzae* pv. *oryzicola* in the above-mentioned virulence-associated functions (Supplemental Figures S4, S5, and S6).

Transcriptional expression of *pgk*, a known virulence-associated gene, was reduced in the *epv*-deletion mutant in both NB and XOM3 medium. It is reported that a number of virulence-associated genes, including *wxolB* (encodes putative glycosyltransferase), *pgk* (encodes phosphoglycerate kinase), *gapA* (encodes glyceraldehydes-3-phosphate dehydrogenase, type I), *tal-C10c-like* (encodes a transcriptional activator-like TAL effector), *dsbC* (encodes disulfide isomerase), *rfaA* (encodes glucose-1-phosphate thymidyltransferase), *atmR* (encodes aminotransferase), *thrC* (encodes threonine synthase), *purF* (encodes amidophosphoribosyltransferase), and *trpA* (encodes tryptophan synthase alpha subunit), were identified by transposon insertion mutagenesis in *X. oryzae* pv. *oryzicola* (8,24). Because mutation of *epv* impaired pathogen virulence but did not affect several important virulence-related functions (such as growth and protease activity), it is reasonable to investigate whether deletion of *epv* impaired or reduced the expression of known virulence-

Fig. 2. Extracellular polysaccharide and virulence-related (*Eps*) protein was involved in extracellular polysaccharide (EPS) production in *Xanthomonas oryzae* pv. *oryzicola*. **A**, EPS production in wild-type strain of *X. oryzae* pv. *oryzicola* and its derivative mutants. **B**, Differential expression of *gumB*, a gene encoding exopolysaccharide xanthan biosynthesis export protein in wild-type strain of *X. oryzae* pv. *oryzicola* and its derivative. Rs105, wild-type strain of *X. oryzae* pv. *oryzicola*; Δepv , *epv*-deletion mutant of *X. oryzae* pv. *oryzicola*; $\Delta hshB$, *hshB*-deletion mutant of *X. oryzae* pv. *oryzicola* (30); Δepv (epv), complemented strain of Δepv . Vertical bars represent standard errors of the means. Different letters above the data bars indicate a significant difference between the strains in A ($P < 0.05$; Fisher's least significant difference test) and B ($P < 0.05$, *t* test), respectively.

associated genes, resulting in the virulence reduction of *epv* mutant. For this purpose, 10 of the known virulence-associated genes described above were selected for the corresponding experiment. We observed that deletion of *epv* significantly reduced the transcriptional expression of *pgk*, *purF*, and *trpA* in the NB broth (nutrient-rich condition) (Fig. 4A). Furthermore, it was shown that transcriptional expression of *pgk*, *gapA*, and *atmR* was obviously reduced in the *epv* mutant compared with the wild-type strain in the XOM3 growth medium (a medium that mimics the intracellular space of plant cell). Combined with these results, it was concluded that the transcriptional expression of *pgk* was reduced in both the nutrient-rich and plant-cell-mimicking medium.

DISCUSSION

In this study, we cloned six unique putative hypothetical secreted protein from *X. oryzae* pv. *oryzicola* compared with *X. oryzae* pv. *oryzae*, and demonstrated for the first time that one of them (*epv*) was required for optimal EPS production and virulence in *X. oryzae* pv. *oryzicola*.

In genome, *Epv* protein was annotated as a hypothetical secreted protein in *X. oryzae* pv. *oryzicola* strain BLS256. However, the results of signal peptide prediction showed that no signal peptide was detected in the amino acid sequence (data not shown), indicating that *Epv* might not be a T2SS-dependent secretion protein. Further Simple Modular Architecture Research Tool (SMART)-based domain analysis (<http://smart.embl-heidelberg.de/>) showed that a transmembrane domain (YFWSWATFFGPVNLV TAAPTFTF) was predicted from position 74 to 96 of *Epv* protein in *X. oryzae* pv. *oryzicola*. This result might provide the clue to explain why *Epv* was defined as a hypothetical secreted protein in the genome of *X. oryzae* pv. *oryzicola*. Moreover, another domain, called low compositional complexity domain (SGGGTSGTGT), was also predicted from position 52 to 62 of *Epv* protein in *X. oryzae* pv. *oryzicola*. This domain referred to the regions containing little diversity in their amino acid composition. The degree of diversity they exhibit might vary, ranging from regions comprising few different amino acids to those comprising just one, the amino acid positions within these regions being either loosely clustered, irregularly spaced, or periodic (7).

Furthermore, a Blastp search showed that a number of *Xanthomonas* spp. (except *X. oryzae* pv. *oryzae*), including *X. cam*

Fig. 3. Extracellular polysaccharide and virulence-related (*epv*) gene transcription is positively regulated by diffusible signal factor (DSF) and global regulator Clp. Differential expression of *epv* was confirmed using relative quantitative real-time polymerase chain reaction. Total RNA was extracted from wild-type strain Rs105 of *Xanthomonas oryzae* pv. *oryzicola*, *ΔrpfF* (DSF-deficient strain), and *Δclp* at the middle stage of growth (optical density at 600 nm = 1.6). Vertical bars represent standard errors of the means. Different letters above the data bars indicate a significant difference between the wild-type strain and *rpfF*-deletion mutant (30) or *clp*-deletion mutant (29) ($P < 0.05$, *t* test).

pestris pv. *musacearum*, *X. campestris* pv. *vesicatoria*, *X. perforans*, *X. fuscans*, *X. axonopodis* pv. *citrumelo*, *X. axonopodis* pv. *citri*, *X. campestris* pv. *campestris*, *X. vesicatoria*, and *X. gardneri*, contain a protein with >95% identical amino acids and size similar to *Epv* found in *X. oryzae* pv. *oryzicola*. Further analysis revealed that the homolog of *Epv* (with >90% identical amino acids) was detected in other bacterial genus, such as *Burkholderia*, *Pseudomonas*, *Methylovorus*, and *Gluconobacter*. Taken together, these results indicated that *Epv* is highly conserved not only in *Xanthomonas* spp. (except *X. oryzae* pv. *oryzae*) but also in other bacteria genera. However, the role or roles of the *Epv* homologue in these bacterial species remained to be investigated.

EPS production is regarded as an important virulence-associated function in *Xanthomonas* spp. Loss of EPS production impairs pathogen virulence in *X. oryzae* (4). In this study, we found that deletion of *epv* significantly reduced the EPS pro-

Fig. 4. Transcription determination of 10 known virulence-associated gene of *Xanthomonas oryzae* pv. *oryzicola* in extracellular polysaccharide and virulence-related (*epv*) gene deletion mutant and wild-type strain. **A**, Comparison of transcriptional expression of 10 known virulence-associated genes of *X. oryzae* pv. *oryzicola* cultivated in nutrient-rich broth (NB) between *epv*-deletion mutant and wild-type strain. Total RNA was extracted from wild-type strain Rs105 of *X. oryzae* pv. *oryzicola* and *Δepv* (*epv*-deletion mutant) at the middle stage of growth (optical density at 600 nm [OD₆₀₀] = 1.6). **B**, Comparison of transcriptional expression of 10 known virulence-associated genes of *X. oryzae* pv. *oryzicola* cultivated in plant-cell-mimicking broth (XOM3) between *epv*-deletion mutant and wild-type strain Rs105. In this assay, *X. oryzae* pv. *oryzicola* strains were preincubated in NB medium overnight, resuspended at OD₆₀₀ = 2.0 in XOM3 medium, and washed twice. Then, 2 ml of the bacterial suspension was inoculated into 50 ml of the plant-cell-mimicking broth XOM3 (pH 6.5) at 28 °C for 16 h (15). Then, total RNA was extracted from wild-type strain Rs105 of *X. oryzae* pv. *oryzicola* and *Δepv* (*epv*-deletion mutant). Vertical bars represent standard errors of the means. Different letters above the data bars indicate a significant difference between the wild-type strain and *epv*-deletion mutant ($P < 0.05$, *t* test).

duction and the transcriptional expression of *gumB* (Fig. 2A and B), which is a key gene encoding exopolysaccharide xanthan biosynthesis export protein. Thus, we assumed that reduction of EPS production might contribute to the virulence reduction in the *epv*-deletion mutant. However, interestingly, the EPS production level of *X. oryzae* pv. *oryzae* carrying pBEPV (broad-host vector pBBR1-MCS5 carried a 920-bp DNA fragment containing an intact *epv* gene and its predicted promoter) was similar to the wild-type *X. oryzae* pv. *oryzae* strain (Supplemental Figure S9). This result indicated that the contribution of Epv protein to EPS production might be different in the two pathovars of *X. oryzae* (pvs. *oryzae* and *oryzicola*).

A recent review demonstrated that EPS had been associated with many bacterial phenotypes, such as biofilm formation and motility (26). In this study, the results showed that mutation of *epv* did not impair the wild-type ability in biofilm formation, motility, and colony morphology in *X. oryzae* pv. *oryzicola*, even though the EPS production of the *epv*-deletion mutant of *X. oryzae* pv. *oryzicola* was impaired (Supplemental Figures S7 and S8), which highly contrasted with the findings in other bacteria, such as in *Pseudomonas aeruginosa* (22). However, the mechanisms of why *epv* did not affect these above-mentioned phenotypes under the EPS-decreasing condition in *X. oryzae* pv. *oryzicola* was still unknown, and need to be further studied.

In this study, we proved that, compared with the wild-type strain, the transcriptional expression of one member (*pgk*, encoding a phosphoglycerate kinase) of known virulence-related genes was reduced in the *epv*-deletion mutant under both nutrient-rich and plant-cell-mimicking growth conditions. This result indicated that reduction of *pgk* might contribute to virulence reduction in the *epv*-deletion mutant.

QS is a signal-dependent cell-to-cell communication mechanism to control bacterial behavior and coordinating gene expression in a cell-density-dependent manner (18). In gram-negative bacteria, the N-acyl homoserine lactones (AHLs) were described as a major class of signal molecules and composed of a homoserine lactone ring carrying C₄-C₁₈ acyl chains (5,17). In the genus *Xanthomonas*, the bacteria does not produce AHL but was able to produce another QS signal, called DSF, which is significantly different from AHLs in chemical structure (1,3,23). Similar to AHL function, the DSF function has been linked to the regulation of virulence, EPS production, and extracellular enzyme (6,12). In this study, *epv* proved to be involved in the EPS production and virulence in *X. oryzae* pv. *oryzicola*, which was consistent with the observation of the role of QS in the same phenotypes in *X. oryzae* pv. *oryzicola*, as previously reported by Wang et al. (27). Thus, these phenotype testing results further confirmed that *epv* was regulated by a DSF-mediating QS system, and indicated that it might be located somewhere in the DSF-signaling pathway.

It is evident that, in the DSF signaling network of *X. campestris* pv. *campestris*, one transcriptional factor, named Clp (cAMP receptor protein-like protein) was regarded as a master regulator and was demonstrated to affect the expression of genes belonging to the DSF regulon such as the genes involved in EPS synthesis (2,10,11). We observed that the transcriptional expression of *epv* was also positively regulated by Clp, indicating that *epv* was located the downstream of Clp. According to the previous study (29), the phenotypes of the *clp*-deletion mutant in EPS production and virulence was similar to the *epv*-deletion mutant presented in this study, which further confirmed that *epv* was located downstream of Clp. Taken together, we conclude that *epv* was regulated by DSF-mediating QS, located downstream of global regulator Clp, and involved in EPS production and virulence in *X. oryzae* pv. *oryzicola*.

Resistance to oxidative stress, protease activity, and growth are regarded as the important virulence-associated functions in *X. oryzae* pv. *oryzicola* (4,8,32). However, in this study, mutation of

epv did not impair these virulence-associated functions, indicating that contribution of Epv protein to virulence was independent of the above-mentioned virulence-associated functions in *X. oryzae* pv. *oryzicola*.

In summary, we showed, for the first time, the novel roles of a putative hypothetical secreted protein (Epv) of *X. oryzae* pv. *oryzicola* in EPS production and virulence. To our knowledge, this was also the first report of genetics and roles of Epv and its homolog in the genus *Xanthomonas*.

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LITERATURE CITED

- Barber, C. E., Tang, J. L., Feng, J. X., Pan, M. Q., Wilson, T. J., Slater, H., Dow, J. M., Williams, P., and Daniels, M. J. 1997. A novel regulatory system required for pathogenicity of *Xanthomonas campestris* is mediated by a small diffusible signal molecule. *Mol. Microbiol.* 24:555-566.
- Büttner, D., and Bonas, U. 2010. Regulation and secretion of *Xanthomonas* virulence factors. *FEMS Microbiol. Rev.* 34:107-133.
- Chatterjee, S., and Sonti, R. V. 2002. *rpfF* mutants of *Xanthomonas oryzae* pv. *oryzae* are deficient for virulence and growth under low iron conditions. *Mol. Plant-Microbe Interact.* 15:463-471.
- Chen, L., Hu, B. S., Qian, G. L., Wang, C., Yang, W. F., Han, Z. C., and Liu, F. Q. 2009. Identification and molecular characterization of twin-arginine translocation system (Tat) in *Xanthomonas oryzae* pv. *oryzae* strain PXO99. *Arch. Microbiol.* 191:163-170.
- Danhorn, T., and Fuqua, C. 2007. Biofilm formation by plant-associated bacteria. *Annu. Rev. Microbiol.* 61:401-422.
- Deng, Y. Y., Wu, J. E., Tao, F., and Zhang, L. H. 2011. Listening to a new language: DSF-based quorum sensing in gram-negative bacteria. *Chem. Rev.* 111:160-173.
- Depristo, M. A., Zilversmit, M. M., and Hartl, D. L. 2006. On the abundance, amino acid composition, and evolutionary dynamics of low-complexity regions in proteins. *Gene* 378:19-30.
- Dharmapuri, S., and Sonti, R. V. 1999. A transposon insertion in the *gumG* homologue of *Xanthomonas oryzae* pv. *oryzae* causes loss of extracellular polysaccharide production and virulence. *FEMS Microbiol. Lett.* 179:53-59.
- Guo, W., Cui, Y. P., Li, Y. R., Che, Y. Z., Yuan, L., Zou, L. F., Zou, H. S., and Chen, G. Y. 2011. Identification of seven genes of *Xanthomonas oryzae* pv. *oryzicola* potentially involved in pathogenesis in rice. *Microbiology*. Online publication. doi:10.1099/mic.0.0050419-0
- He, Y. W., Ng, A. Y., Xu, M., Lin, K., Wang, L. H., Dong, Y. H., and Zhang, L. H. 2007. *Xanthomonas campestris* cell-cell communication involves a putative nucleotide receptor protein Clp and a hierarchical signalling network. *Mol. Microbiol.* 64:281-292.
- He, Y. W., Xu, M., Lin, K., Ng, Y. J., Wen, C. M., Wang, L. H., Liu, Z. D., Zhang, H. B., Dong, Y. H., Dow, J. M., and Zhang, L. H. 2006. Genome scale analysis of diffusible signal factor regulon in *Xanthomonas campestris* pv. *campestris*: identification of novel cell-cell communication-dependent genes and functions. *Mol. Microbiol.* 59:610-622.
- He, Y. W., and Zhang, L. H. 2008. Quorum sensing and virulence regulation in *Xanthomonas campestris*. *FEMS Microbiol. Rev.* 32:842-857.
- Kaufmann, S. H., and Hess, J. 1999. Impact of intracellular location of and antigen display by intracellular bacteria: Implications for vaccine development. *Immunology letters*. *Immunol. Lett.* 65:81-84.
- Kovach, M. E., Elzer, P. H., Hill, D. S., Robertson, G. T., Farris, M. A., Roop, R. M., 2nd., and Peterson, K. M. 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* 166:175-176.
- Lee, S. W., Jeong, K. S., Han, S. W., Lee, S. E., Phee, B. K., Hahn, T. R., and Ronald, P. 2008. The *Xanthomonas oryzae* pv. *oryzae* PhoPQ two-component system is required for AvrXA21 activity, *hrpG* expression, and virulence. *J. Bacteriol.* 190:2183-2197.

16. Li, Y. R., Zou, H. S., Che, Y. Z., Cui, Y. P., Guo, W., Zou, L. F., Chatterjee, S., Biddle, E. M., Yang, C. H., and Chen, G. Y. 2011. A novel regulatory role of HrpD6 in regulating *hrp-hrc-hpa* genes in *Xanthomonas oryzae* pv. *oryzicola*. *Mol. Plant-Microbe Interact.* 24:1086-1101.
17. Miller, M. B., and Bassler, B. L. 2001. Quorum sensing in bacteria. *Annu. Rev. Microbiol.* 55:165-199.
18. Mole, B. M., Baltrus, D. A., Dangl, J. L., and Grant, S. R. 2007. Global virulence regulation networks in phytopathogenic bacteria. *Trends Microbiol.* 15:363-371.
19. Niño-Liu, D. O., Ronald, P. C., and Bogdanove, A. J. 2006. *Xanthomonas oryzae* pathovars: model pathogens of a model crop. *Mol. Plant Pathol.* 7:303-324.
20. Pallen, M. J., Chaudhuri, R. R., and Henderson, I. R. 2003. Genomic analysis of secretion systems. *Curr. Opin. Microbiol.* 6:519-527.
21. Rott, P., Fleites, L., Marlow, G., Royer, M., and Gabriel, D. W. 2011. Identification of new candidate pathogenicity factors in the xylem-invasive pathogen *Xanthomonas albilineans* by transposon mutagenesis. *Mol. Plant-Microbe Interact.* 24:594-605.
22. Ryder, C., Byrd, M., and Wozniak, D. J. 2007. Role of polysaccharides in *Pseudomonas aeruginosa* biofilm development. *Curr. Opin. Microbiol.* 10:644-648.
23. Tang, J. L., Liu, Y. N., Barber, C. E., Dow, J. M., Wootton, J. C., and Daniels, M. J. 1991. Genetic and molecular analysis of a cluster of *rpf* genes involved in positive regulation of synthesis of extracellular enzymes and polysaccharide in *Xanthomonas campestris* pathovar *campestris*. *Mol. Gen. Genet.* 226:409-417.
24. Tjalsma, H., Antelmann, H., Jongbloed, J. D. H., Braun, P. G., Darmon, E., Dorenbos, R., Dubois, J. F., Westers, H., Zanen, G., Quax, W. J., Kuipers, O. P., Bron, S., Hecker, M., and van Dijl, J. M. 2004. Proteomics of protein secretion by *Bacillus subtilis*: separating the "secrets" of the secretome. *Microbiol. Mol. Biol. Rev.* 68:207-223.
25. Vellamy, K. M., Mariappan, V., Hashim, O. H., and Vadivelu, J. 2011. Identification of immunoreactive secretory proteins from the stationary phase culture of *Burkholderia pseudomallei*. *Electrophoresis* 32:210-320.
26. Vu, B., Chen, M., Crawford, R. J., and Ivanova, E. P. 2009. Bacterial extracellular polysaccharides involved in biofilm formation. *Molecules* 14:2535-2554.
27. Wang, L., Makino, S., Subedee, A., and Bogdanove, A. J. 2007. Novel candidate virulence factors in rice pathogen *Xanthomonas oryzae* pv. *oryzicola* as revealed by mutational analysis. *Appl. Environ. Microbiol.* 73:8023-8027.
28. Watt, S. A., Wilke, A., Patschkowski, T., and Niehaus, K. 2005. Comprehensive analysis of the extracellular proteins from *Xanthomonas campestris* pv. *campestris* B100. *Proteomics* 5:153-167.
29. Zhao, Y. C., Qian, G. L., Fan, J. Q., Liu, C. H., Yin, F. Q., Zhou, Y. J., Shen, Q., Hu, B. S., and Liu, F. Q. 2012. Identification and characterization of a novel gene *hshB* in *Xanthomonas oryzae* pv. *oryzicola* co-regulated by quorum sensing and *clp*. *Phytopathology* 102:252-259.
30. Zhao, Y. C., Qian, G. L., Yin, F. Q., Fan, J. Q., Zhai, Z. W., Liu, C. H., Hu, B. S., and Liu, F. Q. 2011. Proteomic analysis of the regulatory function of DSF-dependent quorum sensing in *Xanthomonas oryzae* pv. *oryzicola*. *Microbiol. Pathogenesis* 50:48-55.
31. Zou, L. F., Li, Y. R., and Chen G. Y. 2011. A non-marker mutagenesis strategy to generate poly-*hrp* gene mutants in the rice pathogen *Xanthomonas oryzae* pv. *oryzicola*. *Agric. Sci. China* 10:1139-1150.
32. Zou, L. F., Wang, X. P., Xiang, Y., Zhang, B., Li, Y. R., Xiao, Y. L., Wang, J. S., Walmsley, A. R., and Chen, G. Y. 2006. Elucidation of the *hrp* clusters of *Xanthomonas oryzae* pv. *oryzicola* that control the hypersensitive response in nonhost tobacco and pathogenicity in susceptible host rice. *Appl. Environ. Microbiol.* 72:6216-6224.