The Two-Component System DcuS-DcuR Is Involved in Virulence and Stress Tolerance in the Poplar Canker Bacterium *Lonsdalea populi*

Zeyang Zheng,¹ Chaoying Deng,² Wei He,¹ Wei Qian,² and Aining Li^{1,†}

¹ Beijing Key Laboratory for Forest Pest Control, College of Forestry, Beijing Forestry University, Beijing, China

² State Key Laboratory of Plant Genomics, Institute of Microbiology, Chinese Academy of Sciences, Beijing, China

Accepted for publication 3 June 2020.

ABSTRACT

The gram-negative bacterium *Lonsdalea populi* causes an emerging poplar (*Populus × euramericana*) canker resulting in severe losses to poplar production in China and Europe. Two-component signal transduction systems play important roles in the regulation of virulence and stress responses in phytopathogenic bacteria. We identified a two-component pair (Lqp2625-Lqp2624) in *L. populi*, highly homologous to DcuS-DcuR of *Escherichia coli*. Mutants lacking *DcuS* or *DcuR* displayed normal growth while their virulence on poplar twigs was impaired. An inability to produce flagella indicated that *DcuS* and *DcuR* are involved in biofilm formation and swimming motility. Moreover, the loss of *DcuS* or *DcuR* led to increased sensitivity to oxidative stress and

chloramphenicol through downregulation of genes associated with catalases and the multidrug efflux pump, suggesting that the two-component pair contributes to cellular adaptation to oxidative and antibiotic stresses. We identified key domains and putative phosphorylation sites important for virulence and stress responses. Our findings reveal the functions of DcuS-DcuR in virulence and stress responses in *L. populi* and provide increasing evidence that two-component systems are crucial during the infection process and stress adaptation in bacteria.

Keywords: bacteriology, *Lonsdalea populi*, poplar canker, stress tolerance, two-component system, virulence

Interspecific hybrid poplar *Populus × euramericana* is one of the most widely grown poplars in China. *P. × euramericana* is attacked by many insects and pathogens, including the emerging gram-negative bacterium *Lonsdalea populi* (formerly *Lonsdalea quercina* subsp. *populi*) (Li and He 2019; Li et al. 2014, 2017). This bacterium is responsible for severe bark canker on a large portion of the *P. × euramericana* plantation area in China (Li and He 2019) and in Spain (Berruete et al. 2016). The disease mainly occurs on poplar stems or branches with necrotic lesions and sticky brown-colored fluid oozes, and infested poplars grow weak and eventually die (Li and He 2019). *L. populi* has received great attention in recent years, particularly as it can infect willow trees (*Salix matsudana*) in the wild (Li et al. 2019). However, there is still relatively little knowledge of the molecular mechanism underlying *L. populi* pathogenesis.

Two-component signal transduction systems (TCSs) play important roles in the regulation of physiological and cellular processes in pathogenic bacteria, including pathogenicity (Stock et al. 2000). A typical TCS consists of a transmembrane sensor histidine kinase (HK) and a cytosolic response regulator (RR) (Capra and Laub 2012). The HK is auto-phosphorylated after sensing an environmental or host stimulus, and a phosphoryl group is transferred to the RR, which activates appropriate responses (Urao et al. 2000). Multiple TCSs in bacteria respond to changes in the extracellular environment such as nutrient absorption, redox

state, osmotic pressure, antibiotics, physiological metabolism, quorum sensing, and virulence (Gotoh et al. 2010). For instance, in *Dickeya zeae*, VfmL-VfmH is involved in production of zeamines and cell wall degrading enzymes and in virulence on rice seeds and potato tubers (Lv et al. 2019); PidS/PidR globally regulates pigmentation and virulence-related phenotypes in *Burkholderia glumae* (Karki et al. 2012); ColS-ColR plays an important role in biofilm formation, lipopolysaccharide production, catalase activity, and tolerance to environmental stresses in *Xanthomonas citri* subsp. *citri* (Yan and Wang 2011). In *Escherichia coli*, C4-dicarboxylates serve as source of energy and carbon for aerobic and anaerobic growth and play an important role in the metabolism process. Expression of essential genes involved in C4-dicarboxylates metabolism is regulated by the DcuS-DcuR two-component system (Lukas et al. 2010; Worner et al. 2018). Owing to their dominance in bacterial signal transduction, TCSs are an ideal starting point for elucidating some molecular mechanism of pathogenesis in bacteria.

Whole-genome sequencing shows that *L. populi* has 18 HKs and 24 RRs (Li and He 2019). A large-scale insertional mutagenesis study identified 19 TCS genes involved in bacterial virulence on poplar (Yang et al. 2018). Except for KdpD-KdpE, which plays a crucial role in swimming motility and virulence (Yang et al. 2018), other *L. populi* TCSs have not been well characterized yet. In the present study, we functionally characterized a TCS (Lqp2625-Lqp2624) in *L. populi* that is homologous to DcuS-DcuR of *E. coli* and demonstrated its involvement in biofilm formation, swimming motility, and virulence. We also showed that this TCS contributes to adaptation to oxidative and antibiotic stresses by regulating genes associated with antioxidation and the multidrug efflux pump. Furthermore, we identified key domains and phosphorylation sites affecting the activity of DcuS-DcuR and revealed that these are required for virulence and stress responses. These findings provide further molecular evidence of how TCSs govern virulence and stress responses in *L. populi*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1.

[†]Corresponding author: A. Li; li_aining@bjfu.edu.cn

Funding: This research was supported by the National Natural Science Foundation of China (31870626).

Author contributions: A.L. conceived the experiments. Z.Z. performed the experiments. Z.Z., W.H., and A.L. analyzed the data. A.L. and Z.Z. wrote the manuscript. All authors read and approved the manuscript.

*The e-Xtra logo stands for “electronic extra” and indicates there one supplementary table and two supplementary figures are published online.

The author(s) declare no conflict of interest.

All strains were stored at -80°C with the protection of 40% (vol/vol) glycerol for long-term maintenance. *L. populi* and its derived strains were cultured in Luria-Bertani (LB) medium (per liter: 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl) at 30°C . *E. coli* strains were cultured in LB medium at 37°C . *L. populi* electro-competent cells were prepared in 210 medium (per liter: 8 g of casein enzymatic hydrolysate, 5 g of sucrose, 4 g of yeast extract, 3 g of K_2HPO_4 , and 0.3 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; pH 7.0) and transformed with plasmids constructed as described by Yang et al. (2018). Antibiotics were used to screen positive transformants at the following concentrations: rifampicin (25 $\mu\text{g}/\text{ml}$), ampicillin (100 $\mu\text{g}/\text{ml}$), kanamycin (50 $\mu\text{g}/\text{ml}$), and chloramphenicol (CHL) (25 $\mu\text{g}/\text{ml}$).

Constructions of *dcuS* and *dcuR* mutants and complementation strains. Genomic DNA from *L. populi* N-5-1 was extracted and used as template for PCR amplification. Upstream and downstream fragments of the *dcuS* and *dcuR* genes were first amplified, respectively. The upstream and downstream fragments were then merged by SOE PCR method. Fused PCR products were digested using *Bam*HI and *Xho*I and cloned into pWM91 vector to generate recombinant plasmids. Deletion mutants were constructed as previously described (Yang et al. 2018), and putative mutants were verified by PCR. For complementation strains, fragments including the promoter and open reading frame were amplified, digested using *Hind*III and *Xba*I, cloned into pBBR1MCS2 vector to generate recombinant plasmids and electroporated into competent cells of the respective mutant. The Fast Mutagenesis System (Transgene Biotech, China) was used to introduce point mutations at phosphorylation sites of *dcuS* or *dcuR*. Primers used for mutant and complementation strains construction are listed in Supplementary Table S1.

Bacterial growth curves. *L. populi* and its derived strains were cultured overnight at 30°C in LB medium and cells were diluted to optical density (OD_{600}) = 0.4. Cell suspensions were inoculated 1:100 into fresh LB medium and cultured at 30°C with shaking at 220 rpm/min for 48 h. The OD_{600} of the cultures was measured at 6-h intervals. The assay was performed three times independently.

Assay of sensitivity to metal, high salt, and osmolarity stresses. Bacterial cells were cultured overnight at 30°C in LB medium and diluted to OD_{600} = 0.4. The suspensions were diluted to 1, 10^{-1} , 10^{-2} , 10^{-3} , and 10^{-4} , and 2 μl of each suspension were inoculated onto LB plates containing either 0.1 mM FeCl_3 , 0.15 mM CoCl_2 , 3 mM MnSO_4 , 0.2 mM CuSO_4 , 3% NaCl, 3% KCl, or 16% sorbitol, and incubated at 30°C for 2 days.

Swimming motility assay and flagella observation. Swimming motility was assessed onto semisolid LB medium (0.3% agar) using a toothpick to inoculate bacterial suspension at OD_{600} = 0.4. Plates were incubated at 30°C for 24 h, and the diameter of the swimming zone was measured. Bacterial flagella were observed under a transmission electron microscope (JEM-1400, Japan). The number of flagella were observed using 30 cells for each strain and counted.

Hydrogen peroxide and antibiotic sensitivity assay. An inhibition zone test was performed to evaluate the sensitivity of bacterial strains to H_2O_2 and CHL. Bacterial suspensions were diluted to OD_{600} = 0.4, and 180 μl of suspension, respectively, was mixed with 10 ml of melted LB agar and poured into a plate just before the medium solidified. Sterile filter paper disks impregnated with 5 μl of 5% H_2O_2 and 3 μl of CHL at 2.5 $\mu\text{g}/\text{ml}$ were placed onto the surface of the agar. The diameter of the inhibition zone was measured after 48 h of incubation at 30°C .

Biofilm formation assay. Biofilm formation was quantified by crystal violet assay (Kim et al. 2009). The concentration of bacterial cultures was first adjusted to OD_{600} = 0.4, and then aliquots of 200 μl of the suspensions were placed in a sterile 96-well plate and incubated at 30°C for 48 h. The medium was carefully poured off, and the wells were washed with distilled water and stained with 220 μl of 1.0% crystal violet for 30 min. After 30 min of incubation, the remaining crystal violet solution was removed, and the wells were washed twice with distilled water. When the wells were air-dried, biofilm was dissolved in 240 μl of 95% ethanol. The absorbance of each well was measured using an Infinite 200 Pro microplate reader (Tecan, Switzerland) at 590 nm.

Virulence analysis. Bacterial strains were cultured in LB medium containing appropriate antibiotics at 30°C overnight and OD_{600} was adjusted to 0.4. Twenty-centimeter branches of annual

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description ^a	Resource or reference
Strains		
<i>Lonsdalea populi</i>	Wild-type strain, Rif ^R	Lab collection
<i>Escherichia coli</i> DH5 α	Molecular cloning strain	Lab collection
ΔdcuS	<i>dcuS</i> deletion mutant strain, Rif ^R	This study
ΔdcuR	<i>dcuR</i> deletion mutant strain, Rif ^R	This study
$\Delta\text{dcuS-dcuS}$	Complementary strain of ΔdcuS , containing a pBBR1MCS2:: <i>dcuS</i> vector, Kan ^R	This study
$\Delta\text{dcuR-dcuR}$	Complementary strain of ΔdcuR , containing a pBBR1MCS2:: <i>dcuR</i> vector, Kan ^R	This study
$\Delta\text{dcuS-BV}$	<i>dcuS</i> deletion mutant containing a blank pBBR1MCS2 vector, Kan ^R	This study
$\Delta\text{dcuR-BV}$	<i>dcuR</i> deletion mutant containing a blank pBBR1MCS2 vector, Kan ^R	This study
ΔsCache_3_2	sCache _{3_2} domain deletion mutant of <i>dcuS</i> , Rif ^R	This study
ΔPAS	PAS domain deletion mutant of <i>dcuS</i> , Rif ^R	This study
ΔSPOB_a	SPOB _a domain deletion mutant of <i>dcuS</i> , Rif ^R	This study
$\Delta\text{HATPase}_c$	HATPase domain deletion mutant of <i>dcuS</i> , Rif ^R	This study
<i>dcuS</i> ^{H326A}	Histidine site mutant of <i>dcuS</i> , Rif ^R	This study
<i>dcuR</i> ^{D56A}	Aspartic acid site mutant of <i>dcuR</i> , Rif ^R	This study
Plasmids		
pWM91	Suicide vector to construct mutant of <i>L. populi</i> , Amp ^R	Hoang et al. (1998)
pBBR1MCS2	Genetic complementation vector, Kan ^R	Kessler et al. (1992)
pWM91- <i>dcuS</i>	Deletion vector of <i>dcuS</i> , Amp ^R	This study
pWM91- <i>dcuR</i>	Deletion vector of <i>dcuR</i> , Amp ^R	This study
pWM91-sCache _{3_2}	Deletion vector of sCache _{3_2} mutant, Amp ^R	This study
pWM91-PAS	Deletion vector of PAS mutant, Amp ^R	This study
pWM91-SPOB _a	Deletion vector of SPOB _a mutant, Amp ^R	This study
pWM91-HATPase _c	Deletion vector of HATPase _c mutant, Amp ^R	This study
pBBR- <i>dcuS</i>	pBBR1MCS2:: <i>dcuS</i> , genetic complementation vector, Kan ^R	This study
pBBR- <i>dcuR</i>	pBBR1MCS2:: <i>dcuR</i> , genetic complementation vector, Kan ^R	This study

^a Rif^R, Kan^R, and Amp^R indicate resistance to rifampicin, kanamycin, and ampicillin, respectively.

P. × euramericana seedlings were cut and inoculated with the bacterial cultures. Before inoculation, the inoculation site was washed with distilled water and surface disinfected with 75% alcohol. The branch was carved (1 × 1 cm cross) to expose the xylem and inoculated with 80 µl of bacterial suspension. The wound was wrapped with sterile wet cotton and cling film. Branches were placed in an incubator at 30°C with 90% relative humidity, 12 h of light and 12 h of darkness. The cotton and cling film were removed 3 days after inoculation. The bark was removed 9 days after inoculation and lesion length was scored. Four biological replicates were performed for each strain, and the experiment was repeated three times.

Statistical analysis. Data were expressed as means $\pm$ standard errors. Statistical analyses were performed using one-way analysis of variance (*, $P < 0.05$; and **, $P < 0.01$).

Deletion of *DcuS* or *DcuR* causes defects in *L. populi* virulence. In our previous study, we found that the insertional inactivation of two TCS proteins (Lqp2624 and Lqp2625) resulted in a reduction in virulence (Yang et al. 2018). Systematic functional characterization of the *Lqp2624* and *Lqp2625* genes revealed that they were homologous to DcuS-DcuR of *E. coli* (Fig. 1A and B) with Lqp2624 encoding an RR with a REC domain and Lqp2625 encoding a HK with two transmembrane regions, a sCache_3_2 domain, a PAS domain, a SPOB_a domain and an HATPase_c domain (Fig. 1C and D). We further searched for homologs of Lqp2624 and Lqp2625 in the available bacterial genomes (<https://www.ncbi.nlm.nih.gov/genome/>) and found that Lqp2624 and Lqp2625 showed high similarity to DcuS-DcuR of Enterobacteriales, i.e., *Dickeya* (identity >80%), we herein renamed Lqp2624 and Lqp2625 as DcuR and DcuS, respectively. The genes encoding DcuR and DcuS were adjacent in the genome of *L. populi*, and their cotranscription was confirmed by RT-PCR (Fig. 1E).

using gene replacement and complementation, respectively (Supplementary Fig. S1). The deletion mutants showed comparable growth and colony morphology to the wild type and complementary strains (Supplementary Fig. S2A). One-year-old *P. × euramericana* branches were inoculated with bacterial suspensions ($OD_{600} = 0.4$) and lesion lengths were measured at 9 days postinoculation (dpi). Although $\Delta dcuS$ and $\Delta dcuR$ strains produced disease symptoms on branches, lesions were significantly shorter than those caused by the wild type and complementary strains (Fig. 2). Targeted gene deletion of *DcuR* or *DcuS* confirmed that both genes are required for the infection process of *L. populi* (Fig. 2).

Disruption of *DcuS* or *DcuR* significantly decreases swimming motility of *L. populi*. Swimming capability is thought to be related to bacterial virulence. To investigate whether the absence of *DcuS* and *DcuR* affects bacterial motility, we performed a swimming assay on a semisolid LB medium. After 24 h of cultivation, swimming zones of both $\Delta dcuS$ and $\Delta dcuR$ strains were significantly smaller than those in the wild type, and complementation could restore the swimming defects of the mutants (Fig. 3A and B).

We conceived that the weakened swimming motility of the $\Delta dcuS$ and $\Delta dcuR$ strains might be associated with a functional or structural defect in the flagella. Using transmission electron microscopy, we observed that the $\Delta dcuS$ and $\Delta dcuR$ deletion mutants produced less than one flagellum per cell on average ($>70\%$ cells, $n = 30$), while the wild-type strain N-5-1 and complementary strains developed more than two flagella per cell ($>75\%$ cells, $n = 30$) (Fig. 3C). The numbers of flagella formed by N-5-1 and the complementary strains were significantly different from those formed by the $\Delta dcuS$ and $\Delta dcuR$ deletion mutants (Fig. 3D). Furthermore, we checked the expression levels of genes involved in flagella structure using qRT-PCR. Expression levels of *flgA* and *flgB* were significantly reduced in both mutants compared with

those in N-5-1 (Fig. 3E). Interestingly, we also found that expression levels of *flgC* and *flgD* were greatly reduced in the $\Delta dcuS$ mutant but not in the $\Delta dcuR$ mutant compared with those in N-5-1, and expression levels of *flgE* were comparable in all strains (Fig. 3E).

***DcuS* and *DcuR* are required for tolerance to oxidative and antibiotic stress.** We next explored whether *DcuS*-*DcuR* contributed to the appropriate response to adverse stresses in *L. populi*. The $\Delta dcuS$ and $\Delta dcuR$ mutations did not affect the response to most stresses, such as tolerance to Na^+ , K^+ , sorbitol, Cu^{2+} , Fe^{3+} , Mn^{2+} , and Co^{2+} (Supplementary Fig. S2B). However, the $\Delta dcuS$ and $\Delta dcuR$ mutants were much more sensitive to oxidative (5% H_2O_2) and CHL (2.5 $\mu g/ml$) stress than N-5-1 and complementary strains (Fig. 4A, B, D, and E). We further examined the transcriptional regulation of genes encoding catalases (*ahpC*, *katE*, and *dps*) and components of the multidrug efflux pump (*acrZ*, *mdtC*, and *aaeB*) between the wild type and the *dcuS*-*dcuR* mutants treated with 5% H_2O_2 or CHL at 2.5 $\mu g/ml$, respectively, using qRT-PCR. Expression of *ahpC*, *acrZ*, *mdtC*, and *aaeB* genes was induced by H_2O_2 or CHL in the wild type, whereas the expression levels of these genes were significantly lower in the *dcuS*-*dcuR* mutants (Fig. 4C and F). Unexpectedly, expression of *dps* and *katE* was not altered by treatment with H_2O_2 in the wild type but was obviously increased compared with that in the $\Delta dcuS$ and $\Delta dcuR$ mutants (Fig. 4C and F). These results demonstrated that *DcuS*-*DcuR* is indispensable for controlling responses to H_2O_2 and CHL stresses by regulating the expression of genes associated with autoxidation and the multidrug efflux pump.

***DcuS* and *DcuR* impact biofilm formation.** Biofilm formation is known to protect bacteria against various physical and chemical stresses. Therefore, we next determined whether the $\Delta dcuS$ and $\Delta dcuR$ mutants displayed a deficiency in biofilm formation using a crystal violet assay. The biofilm formation ability

Fig. 2. Virulence is attenuated in *Lonsdalea populi* lacking *DcuS* or *DcuR*. **A**, Virulence assay of *Lonsdalea populi* wild type, deletion mutants ($\Delta dcuS$ or $\Delta dcuR$), complementary strains ($\Delta dcuS$ -*dcuS* or $\Delta dcuR$ -*dcuR*), and controls ($\Delta dcuS$ -BV, $\Delta dcuR$ -BV, and mock) on poplar twigs. Upper panels show disease symptoms on poplar twigs. Lower panels represent necrotic lesions in poplar xylem after peeling bark. Photos were taken at 9 days postinoculation (dpi). **B**, Average lesion size of scabs measured on twigs at 9 dpi. Bars indicate standard deviations of the mean of 30 individual poplar twigs. Asterisks indicate statistically significant differences (*, $P < 0.05$; **, $P < 0.01$).

of the *dcuS-dcuR* mutants was significantly reduced compared with that of the wild type and complementary strains (Fig. 5), suggesting that the DcuS-DcuR system is necessary for biofilm formation.

Dissections of key domains and phosphorylation sites in DcuS-DcuR. To reveal the potential roles of key domains and

phosphorylation sites of DcuS-DcuR in virulence and stress responses, we performed domain induced deletions and point mutations of phosphorylation sites in DcuS-DcuR. Four individual domain deletion mutants (Δ Cache_3_2, Δ PAS, Δ SPOB_a, and Δ HATPase_c) and two mutants (*dcuS*^{H326A} and *dcuR*^{D56A})

Fig. 3. Loss of *DcuS* or *DcuR* impaired swimming motility. **A**, Swimming motility of bacterial strains on semisolid Luria-Bertani medium (0.3% agar). Bacterial cultures were inoculated onto medium using toothpicks and incubated at 30°C for 24 h. **B**, Average diameter of swimming zones. **C**, Flagella of strains observed under a transmission electron microscope. **D**, Numbers of flagella on bacterial cells. For each strain, 30 cells were observed using a transmission electron microscope. **E**, Relative expression of flagellar genes (*flgA*, *flgB*, *flgC*, *flgD*, and *flgE*) among mutants, complementary strains, and wild type estimated by qRT-PCR. Bars are mean $\pm$ standard deviation of three independent experiments. Asterisks indicate statistically significant differences from wild type; *, $P < 0.05$; and **, $P < 0.01$.

conferring point mutations of phosphorylation sites (His326 of *dcuS* and Asp56 of *dcuR*) were obtained. Phenotypic observations showed that $\Delta SPOB\_a$, $\Delta HATPase\_c$, and *dcuS*^{H326A} caused significantly smaller lesions on poplar branches than $\Delta Scache\_3\_2$, ΔPAS , *dcuR*^{D56A}, and the wild type (Fig. 6). Furthermore, except for *ΔdcuS*^{H326}, other mutants did not typically form biofilm resembling the wild type (Fig. 7A and B). However, unexpectedly, these mutants showed similar swimming motility to the wild type (Fig. 7C and D). These results indicated that the different domains and two putative phosphorylation sites of DcuS-DcuR differentially contribute to virulence and biofilm formation of *L. populi*.

Next, we determined whether the domain-deletion and phosphorylation mutants were more sensitive to H₂O₂ or CHL than the wild type, similar to the in-frame deletion mutants. $\Delta SPOB\_a$, $\Delta HATPase\_c$, *dcuS*^{H326A}, and *dcuR*^{D56A} exhibited increased

sensitivity to H₂O₂ or CHL compared with the wild type, whereas the sensitivity of $\Delta Scache\_3\_2$ and ΔPAS was comparable to that of the wild type (Fig. 8A, B, D, and E). We used qRT-PCR to determine whether expression of genes associated with autoxidation and the multidrug efflux pump was altered in these mutants similar to the *dcuS-dcuR* mutants. The expression of *dps*, *katE*, and *ahpC* was consistent with phenotypic observation under H₂O₂ treatment. *dps* and *ahpC* were differentially expressed among $\Delta SPOB\_a$, $\Delta HATPase\_c$, *dcuS*^{H326A}, and *dcuR*^{D56A} in the presence of H₂O₂ (Fig. 8C). Under CHL treatment, expression levels of *acrZ* in all mutants were similar to those in the wild type, expression levels of *aaeB* were significantly reduced in $\Delta HATPase\_c$ compared with those in other mutants, expression levels of *mdtC* were significantly reduced in $\Delta Scache\_3\_2$ and $\Delta HATPase\_c$ compared with other mutants (Fig. 8F). These results indicated that

Fig. 4. *ΔdcuS* and *ΔdcuR* mutants displayed increased sensitivity to oxidative and antibiotic stresses. **A**, Colony phenotypes of strains grown on Luria-Bertani (LB) plates in the presence of 5% H₂O₂. Plates were spot inoculated with the same concentration (optical density (OD)₆₀₀ = 0.4) of bacterial suspensions and incubated at 30°C for 48 h. **B**, Diameters of bacterial growth inhibition zones for H₂O₂. Asterisks indicate a significant difference ($P \leq 0.01$) compared with the wild-type strain. **C**, Relative expression of genes encoding antioxidant factors (*dps*, *ahpC*, and *katE*) in the absence or presence of 5% H₂O₂ among the wild type and mutants. **D**, Bacterial colonies on LB plates with chloramphenicol (CHL) at 2.5 μg/ml. Plates were spot inoculated with bacterial suspensions (OD₆₀₀ = 0.4) and incubated at 30°C for 48 h. **E**, Diameters of bacterial growth inhibition zones for CHL. Asterisk indicates a significant difference ($P \leq 0.01$) compared with the wild-type strain. **F**, Relative expression of genes associated with the multidrug efflux pump (*acrZ*, *mdtC*, and *aaeB*) under treatment with CHL at 2.5 μg/ml among the wild type and mutants. tmRNA was used as an internal control. Each gene was assayed in triplicate. N-Tr, nontreatment; and Tr, treatment.

Fig. 5. Inability of mutants to form biofilm. **A**, Bacterial biofilms measured in a 96-well plate using the crystal violet assay. **B**, Biofilm formation quantified using the ratio between optical density (OD)₅₉₀ and OD₆₀₀. Bars represent the mean of eight biological replicates. Asterisks indicate statistically significant differences (*, $P < 0.05$; and **, $P < 0.01$) compared with the wild-type strain.

Fig. 6. Virulence defects of *dcuS-dcuR* mutants lacking domains or phosphorylation sites. **A**, Virulence assay of the *Lonsdalea populi* wild-type strain and mutants lacking domains or putative phosphorylation sites in the DcuS-DcuR system. Upper panels show disease symptoms on poplar twigs. Lower panels represent necrotic lesions in poplar xylem. **B**, Quantification of lesions on poplar twigs inoculated with bacterial strains. Asterisks indicate statistically significant differences (*, $P < 0.05$; and **, $P < 0.01$) from the wild type.

domains and phosphorylation sites play different roles in the regulation of genes associated with autoxidation and the multidrug efflux pump.

DISCUSSION

We previously characterized a TCS, KdpD-KdpE, which controls bacterial swimming motility, growth, and responses to oxidative and antibiotic stresses (Yang et al. 2018). Through insertional activation, we found two other TCS genes (*Lqp2624* and *Lqp2625*), which encode homologs of DcuS-DcuR. In the present study, we used an in-frame deletion strategy to reveal the roles of DcuS-DcuR in virulence and stress responses of *L. populi*.

A noteworthy finding of this study is that DcuS-DcuR is involved in virulence and stress tolerance in the poplar bacterium *L. populi*. The emerging poplar disease, poplar *Lonsdalea* canker has been threatening *P. × euramericana* plantations in China (Li and He, 2019). However, regulatory mechanisms of *L. populi* pathogenicity have not been fully characterized at the molecular level. Here, we functionally characterized a pair of TCS, DcuS-DcuR, which not only controls virulence and biofilm formation, but also contributes to tolerance to oxidative and CHL stresses.

In *E. coli*, DcuS-DcuR regulates C4-dicarboxylate metabolism (Golby et al. 1999; Kim et al. 2009). DcuS responds to fumarate and other C4-dicarboxylates to activate the phosphorylation of DcuR, which in turn regulates the C4-dicarboxylate transporters DctA and DctB and fumarate reductase FrdABCD (Lukas et al. 2010; Wörner et al. 2018). However, the regulatory function of DcuS-DcuR has not yet been reported in plant bacterial pathogens. DcuS possesses sCache_3_2, PAS, SPOB, and HATPase domains, while DcuR acts as a transcription factor and contains the receiver domain REC and a helix-turn-helix (Abo-Amer et al. 2004; Golby et al. 1999; Kim et al. 2009; Scheu et al. 2012). In *L. populi*, DcuR only has a REC domain; however, DcuR can regulate expression of downstream genes such as *flgA*, *flgB*, *acrZ*, and *dps*. Future studies should investigate whether DcuR functions as a transcription factor or not.

Fig. 7. Mutants lacking domains or putative phosphorylation sites showed impaired swimming motility and biofilm formation. **A**, Swimming motility of mutants on semisolid Luria-Bertani medium (0.3% agar) at 30°C for 24 h. **B**, Quantification of swimming diameters of each mutant. **C**, Biofilm formation measured using a crystal violet assay. **D**, Average biofilm formation ability calculated using the ratio between optical density (OD)₅₉₀ and OD₆₀₀. Asterisks indicate statistically significant differences (*, $P < 0.05$; and **, $P < 0.01$) from the wild type.

We observed normal growth and colony morphology of the *dcuS-dcuR* mutants, excluding growth as a factor causing a reduction in virulence. By contrast, we found that deletion of *DcuS* or *DcuR* resulted in defects in flagella development, biofilm formation, and swimming motility. Flagella and swimming motility are key virulence factors for many pathogenic bacteria (Haiko and Westerlund-Wikstrom 2013; Xu et al. 2019). For instance, in *Xanthomonas axonopodis* pv. *citri*, flagella are required for canker development (Malamud et al. 2011); HupB, a histone-like protein, affects virulence by regulating flagella development, affected virulence resulting from the regulation of flagella development in *Xanthomonas citri* ssp. *citri* (Conforte et al. 2019); and *pilD* and *flgK* mutants show impaired virulence due to lack of flagella in *X. axonopodis* pv. *glycines* (Athinuwat et al. 2018). Transmission electron microscopic observations and qRT-PCR indicated that DcuS-DcuR controls flagellar development, and in turn, possibly impact virulence of *L. populi*. FlhC flagellin is required for flagellar assembly (Haiko and Westerlund-Wikstrom 2013) and is essential for growth and virulence in *Edwardsiella tarda* (He et al. 2012). We speculated that reduced abundance of FlhC in the *dcuS-dcuR* mutants is thus responsible for their impaired virulence.

Interestingly, deletions of domains and pointed mutation of phosphorylation sites in DcuS-DcuR did not affect flagellar development or swimming motility; however, Δ *SpoB_a*, Δ *HATPase_c*, and *dcuS^{H326A}* showed significantly attenuated virulence, suggesting that flagellar development and swimming motility are not the only virulence factors of *L. populi*. A plant-derived burst of reactive oxygen species occurs upon challenge by a bacterial inoculum (Colburn-Clifford et al. 2010; Jamet et al. 2003). We found that the *dcuS-dcuR* mutants were sensitive to H₂O₂ and demonstrated that genes encoding catalases were downregulated in the *DcuS-DcuR* mutants. This may also contribute to the reduced virulence of the Δ *dcuS* and Δ *dcuR* mutants.

Bacterial efflux plays an important role in bacterial multidrug resistance (Poole 2001). As shown in Figure 4F, expression of *acrZ*, *mdtC*, and *aeB* was downregulated in the *dcuS-dcuR* mutants, suggesting that DcuS-DcuR control CHL resistance by regulating genes associated with the multidrug efflux pump. Interestingly, the *dcuS-dcuR* mutants showed no altered sensitivity to metal, salt, and osmotic stresses. These findings suggest that DcuS-DcuR plays an exclusive role in oxidative and antibiotic stress but not other environmental stresses.

We also investigated the relationship between phenotypic defects and putative domains and phosphorylation sites of DcuS-DcuR, identifying key domains and phosphorylation sites that affect the TCS activity. Δ *SpoB_a*, Δ *HATPase_c*, and *dcuS^{H326A}* showed attenuated virulence and were sensitive to H₂O₂. *dcuS* and *dcuR^{D56A}* had reduced biofilm formation and tolerance to CHL stress, while the tolerance of Δ *sCache_3_2* and Δ *PAS* to hydrogen peroxide was not affected. These findings indicate that the different domains and the two putative phosphorylation sites of DcuS-DcuR differentially contribute to virulence and stress response, suggesting that the TCS has a variable repertoire of transduction responses that depends on different stimuli. Accordingly, it remains to be determined how these respective domains or sites in DcuS-DcuR function in oxidative and antibiotic stress.

In summary, our results demonstrated the importance of DcuS-DcuR in virulence and stress responses in the poplar canker bacterium *L. populi*. These findings expand our knowledge of the roles DcuS-DcuR play in plant pathogenic bacteria and lay useful foundations for the elucidation of how *L. populi* produces poplar canker.

ACKNOWLEDGMENTS

We thank Xiangyang Shi (Crop Disease, Pests and Genetics Research Unit, SJVASC, USDA-ARS) for the pre-submission revision of the manuscript.

Fig. 8. Mutants lacking domains or putative phosphorylation sites showed increased sensitivity to hydrogen peroxide and chloramphenicol. **A and D,** Colony phenotypes of strains grown in the presence of 5 μ l of 5% H₂O₂ or 3 μ l of chloramphenicol (CHL) at 2.5 μ g/ml. Plates were spot inoculated with the same concentration optical density ((OD)₆₀₀ = 0.4) of bacterial suspensions at 30°C for 48 h. **B and E,** Inhibition zone diameters of strains. **C and F,** Relative gene expression in the wild type and mutants treated with H₂O₂ or CHL for 30 min determined by qRT-PCR. tmRNA was used as an internal control. Each gene was assayed in triplicate. *, $P < 0.05$; **, $P < 0.01$; N-Tr, nontreatment; and Tr, treatment.

LITERATURE CITED

- Abo-Amer, A. E., Munn, J., Jackson, K., Aktas, M., Golby, P., Kelly, D. J., and Andrews, S. C. 2004. DNA interaction and phosphotransfer of the C4-dicarboxylate-responsive DcuS-DcuR two-component regulatory system from *Escherichia coli*. *J. Bacteriol.* 186:1879-1889.
- Athinuwat, D., Brooks, S., Burr, T. J., and Prathuangwong, S. 2018. Flagella and pili of *Xanthomonas axonopodis* pv. *glycines* are associated with motility, biofilm formation and virulence on soybean. *J. Phytopathol.* 166: 590-600.
- Berruete, I. M., Cambra, M. A., Collados, R., Monterde, A., Lopez, M. M., Cubero, J., and Palacio-Bielsa, A. 2016. First report of bark canker disease of poplar caused by *Lonsdalea quercina* subsp. *populi* in Spain. *Plant Dis.* 100:2159.
- Capra, E. J., and Laub, M. T. 2012. Evolution of two-component signal transduction systems. *Annu. Rev. Microbiol.* 66:325-347.
- Colburn-Clifford, J. M., Scherf, J. M., and Allen, C. 2010. *Ralstonia solanacearum* Dps contributes to oxidative stress tolerance and to colonization of and virulence on tomato plants. *Appl. Environ. Microbiol.* 76: 7392-7399.
- Conforte, V. P., Malamud, F., Yaryura, P. M., Terrones, L. T., Torres, P. S., De Pino, V., Chazarreta, C. N., Gudesblat, G. E., Castagnaro, A. P., Marano, M. R., and Vojnov, A. A. 2019. The histone-like protein HupB influences biofilm formation and virulence in *Xanthomonas citri* ssp. *citri* through the regulation of flagellar biosynthesis. *Mol. Plant Pathol.* 20:589-598.
- Golby, P., Davies, S., Kelly, D. J., Guest, J. R., and Andrews, S. C. 1999. Identification and characterization of a two-component sensor-kinase and response-regulator system (DcuS-DcuR) controlling gene expression in response to C4-dicarboxylates in *Escherichia coli*. *J. Bacteriol.* 181: 1238-1248.
- Gotoh, Y., Eguchi, Y., Watanabe, T., Okamoto, S., Doi, A., and Utsumi, R. 2010. Two-component signal transduction as potential drug targets in pathogenic bacteria. *Curr. Opin. Microbiol.* 13:232-239.
- Haiko, J., and Westerlund-Wikstrom, B. 2013. The role of the bacterial flagellum in adhesion and virulence. *Biology (Basel)* 2:1242-1267.
- He, Y., Xu, T., Fossheim, L. E., and Zhang, X.-H. 2012. FliC, a flagellin protein, is essential for the growth and virulence of fish pathogen *Edwardsiella tarda*. *PLoS One* 7:e45070.
- Hoang, T. T., Karkhoff-Schweizer, R. A. R., Kutchma, A. J., et al. 1998. A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: Application for isolation of unmarked *Pseudomonas aeruginosa* mutants. *Gene* 22:77-86.
- Jamet, A., Sigaud, S., Van de Sype, G., Puppo, A., and Herouart, D. 2003. Expression of the bacterial catalase genes during *Sinorhizobium meliloti*-*Medicago sativa* symbiosis and their crucial role during the infection process. *Mol. Plant-Microbe Interact.* 16:217-225.
- Karki, H. S., Barphagha, I. K., and Ham, J. H. 2012. A conserved two-component regulatory system, PidS/PidR, globally regulates pigmentation and virulence-related phenotypes of *Burkholderia glumae*. *Mol. Plant Pathol.* 13:785-794.

- Kessler, B., Lorenzo, V., and Timmis, K. N. 1992. A general system to integrate lacZ fusions into the chromosomes of gram-negative eubacteria: Regulation of the Pm promoter of the TOL plasmid studied with all controlling elements in monocopy. *Mol. Gen. Genet.* 233:293-301.
- Kim, O. B., Reimann, J., Lukas, H., Schumacher, U., Grimpo, J., Dunnwald, P., and Uden, G. 2009. Regulation of tartrate metabolism by TtdR and relation to the DcuS-DcuR-regulated C4-dicarboxylate metabolism of *Escherichia coli*. *Microbiology* 155:3632-3640.
- Li, A., and He, W. 2019. Molecular aspects of an emerging poplar canker caused by *Lonsdalea populi*. *Front. Microbiol.* 10:2496.
- Li, Y., He, W., Ren, F. J., Guo, L. M., Chang, J. P., Cleenwerck, I., Ma, Y. C., and Wang, H. M. 2014. A canker disease of *Populus × euramericana* in China caused by *Lonsdalea quercina* subsp. *populi*. *Plant Dis.* 98: 368-378.
- Li, Y., Wang, H. M., Chang, J. P., Guo, L. M., Yang, X. Q., and Xu, W. 2019. A new bacterial bark canker disease of *Salix matsudana* caused by *Lonsdalea populi* in China. *Plant Dis.* 103:2467.
- Li, Y., Xue, H., Guo, L. M., Koltay, A., Palacio-Bielsa, A., Chang, J., Xie, S., and Yang, X. 2017. Elevation of three subspecies of *Lonsdalea quercina* to species level: *Lonsdalea britannica* sp. nov., *Lonsdalea iberica* sp. nov. and *Lonsdalea populi* sp. nov. *Int. J. Syst. Evol. Microbiol.* 67:4680-4684.
- Lukas, H., Reimann, J., Bin Kim, O., Grimpo, J., and Uden, G. 2010. Regulation of aerobic and anaerobic D-malate metabolism of *Escherichia coli* by the LysR-type regulator DmlR (YeaT). *J. Bacteriol.* 192:2503-2511.
- Lv, M. F., Hu, M., Li, P., Jiang, Z. D., Zhang, L. H., and Zhou, J. N. 2019. A two-component regulatory system VfmIH modulates multiple virulence traits in *Dickeya zeae*. *Mol. Microbiol.* 111:1493-1509.
- Malamud, F., Torres, P. S., Roeschlin, R., Rigano, L. A., Enrique, R., Bonomi, H. R., Castagnaro, A. P., Marano, M. R., and Vojnov, A. A. 2011. The *Xanthomonas axonopodis* pv. *citri* flagellum is required for mature biofilm and canker development. *Microbiology* 157:819-829.
- Poole, K. 2001. Multidrug resistance in Gram-negative bacteria. *Curr. Opin. Microbiol.* 4:500-508.
- Scheu, P. D., Witan, J., Rauschmeier, M., Graf, S., Liao, Y. F., Ebert-Jung, A., Basche, T., Erker, W., and Uden, G. 2012. CitA/CitB two-component system regulating citrate fermentation in *Escherichia coli* and its relation to the DcuS/DcuR system in vivo. *J. Bacteriol.* 194:636-645.
- Stock, A. M., Robinson, V. L., and Goudreau, P. N. 2000. Two-component signal transduction. *Annu. Rev. Biochem.* 69:183-215.
- Urao, T., Miyata, S., Yamaguchi-Shinozaki, K., and Shinozaki, K. 2000. Possible His to Asp phosphorelay signaling in an Arabidopsis two-component system. *FEBS Lett.* 478:227-232.
- Wörner, S., Surmann, K., Ebert-Jung, A., Volker, U., Hammer, E., and Uden, G. 2018. Cellular concentrations of the transporters DctA and DcuB and the sensor DcuS of *Escherichia coli* and the contributions of free and complexed DcuS to transcriptional regulation by DcuR. *J. Bacteriol.* 200: e00612-e00617.
- Xu, J., Koyanagi, Y., Isogai, E., and Nakamura, S. 2019. Effects of fermentation products of the commensal bacterium *Clostridium ramosum* on motility, intracellular pH, and flagellar synthesis of enterohemorrhagic *Escherichia coli*. *Arch. Microbiol.* 201:841-846.
- Yan, Q., and Wang, N. A. 2011. The ColR/ColS two-component system plays multiple roles in the pathogenicity of the citrus canker pathogen *Xanthomonas citri* subsp. *citri*. *J. Bacteriol.* 193:1590-1599.
- Yang, R., Deng, C., Wei, J., He, W., Li, A., and Qian, W. 2018. A large-scale mutational analysis of two-component signaling systems of *Lonsdalea quercina* revealed that KdpD-KdpE regulates bacterial virulence against host poplar trees. *Mol. Plant-Microbe Interact.* 31:724-736.