The Ribosomal Protein RplY Is Required for *Pectobacterium carotovorum* Virulence and Is Induced by *Zantedeschia elliotiana* Extract

Huan Jiang, Mengyi Jiang, Liuke Yang, Peiyan Yao, Lin Ma, Chunting Wang, Huan Wang, Gouliang Qian, Baishi Hu, and Jiaqin Fan†

Department of Plant Pathology, College of Plant Protection, Nanjing Agricultural University, Nanjing, 210095, China.
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ABSTRACT

Pectobacterium carotovorum subsp. *carotovorum* strain PccS1, a bacterial pathogen causing soft rot disease of *Zantedeschia elliotiana* (colored calla), was investigated for virulence genes induced by the host plant. Using a promoter-trap transposon (*mariner*), we obtained 500 transposon mutants showing kanamycin resistance dependent on extract of *Z. elliotiana*. One of these mutants, PM86, exhibited attenuated virulence on both *Z. elliotiana* and *Brassica rapa* subsp. *pekinensis*. The growth of PM86 was also reduced in minimal medium (MM), and the

reduction was restored by adding plant extract to the MM. The gene containing the insertion site was identified as *rplY*. The deletion mutant $\Delta rplY$ exhibited reduced virulence, motility and plant cell wall-degrading enzyme production but not biofilm formation. Analysis of gene expression and reporter fusions revealed that the *rplY* gene in PccS1 is up-regulated at both the transcriptional and the translational levels in the presence of plant extract. Our results suggest that *rplY* is induced by *Z. elliotiana* extract and is crucial for virulence in *P. carotovorum* subsp. *carotovorum*.

Pectobacterium carotovorum subsp. *carotovorum* is a soilborne facultative anaerobic phytopathogenic bacterium. It has a global distribution and causes soft rot in a wide range of crops and ornamental plants during cultivation, transportation, and storage (Fan et al. 2011b; Joshi et al. 2016a; Kubheka et al. 2013; Ma et al. 2007; Mansfield et al. 2012; Potrykus et al. 2014; Tian et al. 2015; Toth et al. 2003). Like other soft-rot Enterobacteriaceae (SREs), including *P. atrosepticum* and *Dickeya dadantii*, *P. carotovorum* subsp. *carotovorum* is a well-studied producer of plant cell wall-degrading enzymes (PCWDEs) (Toth and Birch 2005). PCWDEs are secreted mainly by the type II secretion system (T2SS), and several related T2SS regulators have been identified (Charkowski et al. 2012; Hassan et al. 2013; Kazemi-Pour et al. 2004; Li et al. 2010; Lu et al. 2011; Rodionov et al. 2004; Williamson et al. 2010; Yamazaki et al. 2011). SREs have also been shown to possess the effector protein DspE, which is secreted through the type III secretion system (T3SS) to manipulate the host plant defense response and aid infection (Hogan et al. 2013; Jin et al. 2016).

Previous studies revealed that SREs also respond to plant components and small molecules (Babujee et al. 2007; Charkowski et al. 2012; Joshi et al. 2016b; Kelemu and Collmer 1993; Mattinen et al. 2007; Mehta and Rosato 2001). Four LacI family genes (*lfaR*, *lfcR*, *lfdR*, and *lfeR*) in *D. dadantii* 3937 were found to be important for infection (Van Gijsegem et al. 2008). Moreover, the expression of the adjacent genes controlled by *LfaR* and *LfgR* was specifically induced when the culture medium was supplemented with crude plant extract (Van Gijsegem et al. 2008). The application of functional genomics and proteomics to plant pathogens and their hosts provides a unique opportunity to identify important proteins underlying plant pathogenesis (Babujee et al. 2007; Joshi et al. 2016a; Mattinen et al. 2007; Mehta et al. 2008; Quirino et al. 2010; Rodionov et al. 2004; Wang et al. 2016). Nevertheless, the initial

interaction between plant pathogens of SREs and their host plants remains only partially understood.

To identify bacterial genes that are activated by the plant during the course of their interaction, Cai et al. (2009) constructed a *mariner* transposon carrying a promoterless kanamycin resistance gene to screen for potential genes induced in *Mesorhizobium tianshanense* by plant extracts. The gene *misA*, encoding a putative LysE family translocator, was identified as being activated by legume extracts. Canavanine was further identified as the inducing compound in plant extracts (Cai et al. 2009).

In recent years, colored calla (*Zantedeschia* spp.) is becoming one of the top-ranked ornamental plants worldwide (Fan et al. 2011b; Snijder et al. 2004b). However, due to a lack of effective control measurements, soft rot caused by *P. carotovorum* subsp. *carotovorum* is still a major disease on colored calla, and prevents the worldwide development of this ornamental plant (Snijder et al. 2004a). To identify virulence-associated genes induced by plant extract, we constructed a *mariner* transposon insertion mutant library of the *P. carotovorum* subsp. *carotovorum* strain PccS1, which was isolated from diseased colored calla (*Z. elliotiana*) cultivar ‘Black Magic’ plants in Nanjing (Gu et al. 2009). We characterized an essential gene *rplY* encoding ribosomal protein L25, which was activated by *Z. elliotiana* extract. The phenotypic results showed that *rplY* contributes to virulence of PccS1 which, to our knowledge, is the first time *rplY* has been described in the pathogenesis of *P. carotovorum* subsp. *carotovorum*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth condition. All bacterial strains and plasmids used in this study are listed in Table 1. *P. carotovorum* subsp. *carotovorum* strain PccS1 and its derivatives were maintained on 2YT (tryptone at 17 g·liter⁻¹, NaCl at 5 g·liter⁻¹, and yeast extract at 10 g·liter⁻¹, pH=7.0) or minimal medium (hereafter MM) (Kersey et al. 2012) at 28°C, and *Escherichia coli* was cultured on Luria-Bertani (LB) medium (Wang et al. 2016) or LB agar (LA) plates at 37°C. When required, antibiotics were supplemented at the following final concentrations: chloramphenicol (Cm) at 100 µg·ml⁻¹, kanamycin (Km) at 40 µg·ml⁻¹, rifampin (Rif) at 100 µg·ml⁻¹, gentamicin (Gm) at 100 µg·ml⁻¹, and ampicillin (Amp) at 100 µg·ml⁻¹.

†Corresponding author: J. Fan; E-mail: fanjq@njau.edu.cn

The first two authors contributed equally to this research.

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Plant extract of yellow-flowered *Z. elliotiana* ('Black Magic') was prepared as previously described (Wang et al. 2016). Briefly, the leaves of 7- to 8-week-old *Z. elliotiana* potted plants growing in the greenhouse (temperature varying from 22 to 28°C, 16 h day length and approximately 80% relative humidity) were collected. After being surface-sterilized with 75% ethanol, 10 g of leaf tissue was ground with 40 ml of sterilized water in a blender. The mixtures were adjusted to a final volume of 50 ml with sterilized water. The samples were then filtered twice through gauze (Yierkang, Nanchang, China). The filtrates were centrifuged at 4,000 × *g* for 20 min. To ensure that the plant extract constituents remained chemically similar to their in planta status, supernatants were filter sterilized by passage through a 0.22-μm filter (Dongkang, Tianjin, China) to remove bacteria, and followed by ultra-filtration (Millipore, Red Bank, NJ) twice to eliminate proteins larger than 5 kDa (Mattinen et al. 2007). Finally, the filtrates containing 20% plant extract (wt/vol) were filter sterilized again, dispensed into approximately 2-ml aliquots and stored at -20°C. After conducting pretests, 2YT and MM containing 3% (wt/vol) *Z. elliotiana* leaf extract (2YT + ZE and MM + ZE, respectively) were used in subsequent tests.

Construction of a transposon insertion mutant library and screen for virulence-attenuated mutants. A transposon-tagging insertional mutant library of PccS1 was constructed as described previously (Cai et al. 2009; Judson and Mekalanos 2000). Briefly, *E. coli* strain SM10λpir, containing plasmid pJZ260, with Cm resistance and a promoterless kanamycin resistance transposon, was used as the donor strain. The rifampin-resistant PccS1 strain was used as the recipient in biparental mating. Transconjugants with constitutive and plant extract-induced Km resistant (Km^r) were obtained on 2YT plates containing 3% ZE (wt/vol), Cm, Rif and Km. All the transconjugants exhibiting Km^r were then patched in parallel onto two plates (2YT containing Km, Cm and Rif with or without plant extract) to select plant extract-induced Km^r mutants. The virulence of these mutants was tested as described below. Finally, the candidates were confirmed by PCR with the primers designed according to the specific gene sequences of the transposon and PccS1.

Identification of genes in the plant extract-induced Km^r and virulence-attenuated mutants. The transposon insertion

sites of mutant strains were identified as previously described (Judson and Mekalanos 2000). The plant extract-dependent transposon random-insertion mutants with attenuated virulence were separately digested with *Sa*II. The fragments were ligated into *Sa*II-digested pUC19 and then transformed into the *E. coli* strain DH5α. Transformants were selected on LA plates containing Cm. The colonies were then confirmed by PCR using the transposon tagging primers Km-1 and 137-1, and positive PCR products were sequenced. The sequences were analyzed by BLASTn of the NCBI database.

Genetic manipulation. Genetic manipulations, including gene deletion and complemented strain of the mutation, were performed as previously described (Li et al. 2010; Wang et al. 2016). Briefly, primers were designed according to the sequences of the flanking regions of the target genes. Fragments upstream and downstream of the gene were ligated with the Km^r gene fragment amplified from pET30 (Table 1), and then recycled and cloned into pEX18GM (Table 1) to yield pEX-*rplY* for gene disruption. Plasmid pEX-*rplY* was further cloned into the *E. coli* strain BW20676 to generate a donor strain. With the wild-type strain of PccS1 used as the recipient, transconjugants were constructed by homologous recombination. To select candidate disruption mutants, transconjugants were plated onto 2YT containing 10% sucrose (Solarbio, Beijing, China) and Rif at 100 μg ml⁻¹, Km at 40 μg ml⁻¹, and Gm at 100 μg ml⁻¹. Colonies with resistance to sucrose and kanamycin were isolated. To construct the complemented strains, the target gene and its promoter region were amplified and cloned into a broad-host-range vector pBBR1-MCS5 (Table 1). The generated vector pBBRCp-*rplY* (Table 1) was introduced into each respective mutant by conjugation as described above. The complemented strains were selected based on Gm resistance. All constructs were verified by PCR and sequencing. The complemented strains, carrying the target gene fusion with an epitope tagging fragment (DYKDDDDK) at the C-terminus, were additionally confirmed by western immunoblotting for the expected FLAG-tagged protein (de Bruin et al. 2011). The chromosomal *rplY::lacZ* transcriptional fusion was constructed by cloning the promoter region of *rplY* into pVIK112, and the resulting construct was then transferred into PccS1 by conjugation using *E. coli* BW20676 (Cai et al. 2009). The positive

TABLE 1. Strains and plasmids used in this study

Strains and plasmids	Relevant genotype or description	Source
Strains		
<i>Escherichia coli</i>		
SM10λpir	Conjugal donor strain, Sm ^r , host of pJZ260, Amp ^r , Cm ^r	TakaRa
DH5α	Standard cloning host	TakaRa
BW20676	Conjugal donor strain, host of pVIK112	TakaRa
<i>Pectobacterium carotovorum</i>		
subsp. <i>carotovorum</i>		
PccS1	Wild-type, Rif ^r , isolated from rotted tissue of yellow flowered calla <i>Zantedeschia elliotiana</i> in Nanjing, China	(Gu et al. 2009)
<i>rplY::km</i> (PM86)	Derivative of PccS1 carrying <i>mariner</i> transposon insertion, Rif ^r , Cm ^r , plant-extract-dependent Km ^r	This study
Δ <i>rplY</i>	<i>rplY</i> deletion mutant strain of PccS1, Rif ^r , Km ^r	This study
Δ <i>rplY</i> (<i>rplY</i>)	<i>rplY</i> complemented strain of Δ <i>rplY</i> , Rif ^r , Km ^r , Gm ^r	This study
Δ <i>rplY</i> (pBBR)	pBBR1-MCS5 complemented strain of Δ <i>rplY</i> , Rif ^r , Km ^r , Gm ^r	This study
PccS1(<i>rplY</i> -F)	pBBRCp- <i>rplY</i> -F complemented strain of PccS1, Rif ^r , Gm ^r	This study
Δ <i>rplY</i> (<i>rplY</i> -F)	pBBRCp- <i>rplY</i> -F complemented strain of Δ <i>rplY</i> , Rif ^r , Km ^r , Gm ^r	This study
PccS1(<i>rplY::lacZ</i>)	Transcriptional fusion strain of PccS1 with pVIK112- <i>rplY::lacZ</i>	This study
Plasmids		
pJZ260	R6K vector with a <i>mariner</i> transposon, promoterless, Cm ^r , Km ^r , Amp ^r	(Cai et al. 2009)
pUC19	Amp ^r , ColE1 origin	TaKaRa
pET30	Template plasmid for kanamycin cassette, Km ^r	TaKaRa
pEX18GM	Suicide vector with a <i>sacB</i> gene, Gm ^r	(Hoang et al. 1998)
pVIK112	Suicide vector with a <i>R6k</i> gene, Km ^r	TaKaRa
pVIK112- <i>rplY::lacZ</i>	pVIK112 carrying the promoter of <i>rplY</i> fusion to <i>lacZ</i> , Km ^r	This study
pBBR1-MCS5	Broad host range vector with a P _{lac} Promoter, Gm ^r	(Kovach et al. 1995)
pEX- <i>rplY</i>	Gm ^r , pEX18GM with 2.8-kb fragment of <i>rplY</i>	This study
pBBRCp- <i>rplY</i>	Gm ^r , pBBR1-MCS5 with 1.5-kb fragment of <i>rplY</i>	This study
pBBRCp- <i>rplY</i> -F	Gm ^r , pBBR1-MCS5 with 1.5-kb fragment of <i>rplY</i> carried a C-terminal FLAG epitope tag	This study

strains were verified by PCR assays targeting *rplY*-promoter-1 and *LacZ*-2 and DNA sequencing.

Quantitative real-time PCR (qRT-PCR). The transcript levels of the target genes were measured by qRT-PCR as previously described (Kersey et al. 2012; Wang et al. 2016). Briefly, bacteria were cultured in LB from a 1% dilution of overnight cultures originating from a single colony and harvested by centrifugation when the OD₆₀₀ value reached ~1.0. To determine whether the gene was induced by components of plant extract, cells of the wild-type PccS1 strain cultured both in MM and in MM + ZE for 4, 8, 12, 16, and 20 h were also harvested by the same method. After washing with RNA-free water three times, total RNA was extracted separately using the E.Z.N.A. Bacterial RNA Kit (OMEGA, Norcross, GA). Two milligrams of RNA was then used to synthesize cDNA according to the manufacturer's instructions for the PrimeScript RT Reagent (Perfect Real Time) Kit (TaKaRa, Dalian, China). qRT-PCR was performed in an ABI 7500 Sequence Detection System (Applied Biosystems, Carlsbad, CA) using iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA). The gene-specific primers, including genes for virulence factors and other genes of interest, are listed in Supplementary Table S1. The housekeeping gene, *recA*, was used as an endogenous control for assessing expression (Kersey et al. 2012; Li et al. 2010). Three independent biological experiments were carried out with at least three replicates in each experiment. The data were analyzed by the ABI 7500 software (v.2.0.6).

β-galactosidase activity assay. Cells containing the *rplY::lacZ* transcriptional fusion were cultured in MM and MM + ZE for 20 h at 28°C until the stationary phase was reached (OD₆₀₀ = 2.0). These cultures were used for a β-galactosidase activity assay, as previously described (Cai et al. 2009), using the β-galactosidase assay kit (GENMED Scientifics Inc., Shanghai, China) with O-nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate. All experiments were conducted three times with at least five replicates in each assay.

Western blot analysis. To visualize the expression of the target gene induced by *Z. elliotiana* plant extract, the complemented strain of PccS1 carrying the target gene fusion, with an epitope tagging fragment (DYKDDDDK) at the C-terminus, was blotted as previously described (Sonnleitner and Blasi 2014). Cells were cultured overnight in 2YT at 28°C, centrifuged, pelleted and washed with sterilized water. Cells were then resuspended and diluted 1:100 in 50 ml of fresh MM and MM + ZE, and then cultured at 28°C for 4, 8, 12, 16, and 20 h. Twenty-five milliliters of the cultures were then collected and pelleted by centrifugation (4,000 × *g*) for 5 min. The pellet was then washed with PBS (pH 7.0) three times, and resuspended in radioimmunoprecipitation assay (RIPA) lysates (1% Triton X-100, 1% deoxycholate, 0.1% SDS, added at 5 μl per mg of cells). Phenylmethanesulfonyl fluoride, protease inhibitors, and phosphatase inhibitors were added to the samples at a ratio of 1:100 (vol/vol) of RIPA, and the mixtures were incubated on ice for 3 h. The supernatants were obtained after centrifugation at 4,000 × *g* for 10 min and dispensed for further analysis. An Easy II Protein Quantitative Kit (BCA) (TRANSGEN, Beijing, China) was used to adjust the total protein of each sample to the same level. In accordance with the manufacturer's instructions, the assay was conducted in 96-well microplates as follows: a 5-μl sample was added to 200 μl of BCA working reagent and the plates were incubated at 37°C for 30 min. The absorbance of the samples was then analyzed at 595 nm. For the western blot assay, samples were boiled for 10 min with 4× Protein SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) Loading Buffer (TaKaRa, Dalian, China). Twenty microliters of each sample was loaded onto a 12% SDS-polyacrylamide gel (Bio-Rad), transferred to a nitrocellulose membrane (Bio-Rad), and then transferred to a PVDF membrane (Millipore, Red Bank, NJ). The membranes were then blocked with 5% milk in Tris-buffered saline containing Tween 0.05% (TBST, pH = 7.5), and incubated with a 1:5,000 dilution of the primary DYKDDDDK-Tag (3B9) Mouse mAb (Abmart, Berkeley Heights, NJ) for 1 h. The membrane was then washed twice with TBST and incubated with a 1:10,000 dilution of Goat Anti-Mouse IgG-HRP

(Abmart) for 1 h, and washed three times with TBST. Peroxidase was detected using the ECL Western Blotting Substrate (Bio-Rad). Anti-RNA polymerase (anti-RNAP) polyclonal antibody (Institute of Microbiology, Chinese Academy of Science, Beijing, China) was used as the loading control (Deng et al. 2014).

Phenotypic assays. The plant virulence assay was conducted on leaves as previously described (Li et al. 2010; Wang et al. 2016). Briefly, PccS1 and its derivative strains were cultured overnight in 2YT at 28°C. The cells were harvested and suspended in sterilized water or 3% ZE plant extract to OD₆₀₀ 1.2 (10⁸ CFU ml⁻¹) for inoculation. Approximately 45-day-old leaves were detached from *Z. elliotiana*, *Z. aethiopica*, and Chinese cabbage (*Brassica rapa* subsp. *pekinensis*) plants grown in a greenhouse. The detached leaves were washed thoroughly with sterilized water and surface-sterilized with 75% ethanol. For each site, the abaxial surface of the leaves was punctured with a sterilized tooth pick, and 10 μl of bacterial suspension was applied in parallel onto the wounds. Sterilized water was inoculated as a negative control. The leaf pieces were then incubated in a moist chamber at 28°C, and the maceration area (cm²) on the leaves was measured at 16 h after inoculation (HAI). The virulence assays were conducted three times using 5 to 10 leaf pieces in each assay.

To assess the colonization of the strains, 10 μl of the diluted cells of the appropriate cultures of the wild-type and mutant PccS1 strains was inoculated onto the leaves of *Z. elliotiana* as described above. The macerated tissues (~0.5 × 0.5 cm) were detached from the leaves, weighed, and ground in sterilized water at a volume of 1 ml. The cell suspension from the rotted tissue was subjected to 10-fold serial dilution and patched onto three LA replica plates. After incubation at 28°C for 16 h, colonies were counted. The experiment was repeated three times, and the CFU per gram of rotted tissue was quantified.

Growth curves were established as follow. Cells of PccS1 and its derivatives were cultured overnight, harvested, and suspended in sterilized water to an OD₆₀₀ of 0.6. Cell suspensions were then diluted to a concentration of 1% (vol/vol) in 50 ml of the appropriate medium without antibiotics, and the OD₆₀₀ value was monitored at four hour intervals until stationary phase with a Beckman DU-640 (Beckman, Houston, TX).

To examine the pleiotropic effects of *rplY* on the virulence of PccS1, a number of phenotypes were investigated. Motility was tested by measuring the area (mm²) of swimming haloes on a 2YT plate (containing 0.3% agar) 24 h after 2.5 μl of the cell suspension (in sterilized water adjusted to OD₆₀₀ ~1.0) was spotted onto the plate (Fan et al. 2011a; Wang et al. 2009). Motility was also monitored by observing the sedimentation of the cells in stationary glass tubes for 24 h at room temperature after shaking the culture in 2YT at 28°C until the OD₆₀₀ reached 2.0.

Biofilm development on an abiotic surface was quantified using crystal violet, as previously described (O'Toole and Kolter 1998; Zeng et al. 2013). Briefly, cells were cultured in 2YT broth in tubes at 28°C until the OD₆₀₀ reached 2.0. The cultures were then removed, 1% crystal violet was added to them at the same volume as the cultures. The inner walls of the tubes were then stained for 1 h at room temperature, rinsed three times with sterilized water and air-dried for 2 h. Tubes were photographed and crystal violet was then eluted with 1 ml of 100% ethanol. The crystal violet elution was quantified by measuring the light absorbance at 575 nm.

The PCWDE activity of the wild-type PccS1 and derived mutants was determined. Overnight cultures were harvested by centrifugation and diluted with LB broth to a concentration of 2.0 at OD₆₀₀. A total of 2.5 μl of the diluted cell suspension was spotted onto protease assay plates (LA medium supplemented with 10% sterilized skimmed milk), as well as pectate lyase and cellulase assay plates, as previously described (Barnard et al. 2010). PCWDE production was visualized on the plates after cells were spotted onto the plates and incubated for 48 h at 28°C. Positive colonies on the protease activity assay plates exhibited clear haloes on an opaque white background. Pectate lyase assay plates were stained with 7.5% (wt/vol) copper acetate for 1 h, and positive colonies were identified based on the presence of double cream haloes

on a translucent blue-green background. Cellulase activity assay plates were flooded with 0.2% (wt/vol) Congo red for 20 min, bleached with 1 M NaCl for 15 min, and then stained with 1 M HCl for 5 min. Positive colonies were recognized based on the presence of off-white haloes on a dark blue background. Finally, the areas (mm²) of the haloes were measured. All phenotypic assays were conducted three times with at least five replicates per independent experiment.

Data were statistically analyzed using SPSS 14.0 (SPSS Inc., Chicago, IL). The hypothesis test of percentages (Duncan's multiple

range test, $P = 0.05$ or 0.01) was used to determine significant difference in the assays described above.

RESULTS

Only one *mariner* insertion mutant showed both plant extract-dependent Km^r and attenuated virulence. To identify genes that were activated by *Z. elliotiana* plant extract and related to virulence, a promoter-trap transposon was introduced into

Fig. 1. Alignment of deduced amino acid sequences of *Pectobacterium carotovorum* subsp. *carotovorum* strain PccS1 RplY with homologs from other pathogenic bacteria in the families Enterobacteriaceae and Vibrionaceae in accordance with NCBI (National Center for Biotechnology Information). *P. caro_PccS1*, *P. caro_WPP14*, and *P. caro_PCC21* are strains PccS1, WPP14 and PCC21 of *P. carotovorum* subsp. *carotovorum*, respectively. *P. aro_PC1* is *P. aroidearum* strain PC1. *P. bra_PBR1692* is *P. brasiliense* strain PBR1692. *P. wa_WPP163*, *P. wa_SCC3193*, and *P. wa_3304* are strains WPP163, SCC3193, and 3304 of *P. wasabiae*, respectively. *P. atro_SCR11043* is *P. atrosepticum* strain SCR11043. *D. dad_Ech586* is *Dickeya dadantii* strain Ech586. *E. coli_MG1655* is *Escherichia coli* str. K-12 substr. MG1655, and *V. cho_V51* is *Vibrio cholerae* strain V51. Amino acids highlighted in black are those which are conserved in RplY of PccS1 as well as other pathogenic bacteria strains in the families Enterobacteriaceae and Vibrionaceae.

Fig. 2. Growth curves of *Pectobacterium carotovorum* subsp. *carotovorum* wild-type PccS1 (WT) and the derivative strains in minimal medium (MM): $\Delta rplY$ is the gene deletion mutant; $\Delta rplY(rplY)$ is the complemented strain of $\Delta rplY$ containing a fragment of *rplY*; PM86 is the *mariner* transposon insert mutant. +ZE indicates the cells cultured in MM supplemented with 3% (wt/vol) leaf extract of the *Zantedeschia elliotiana* 'Black Magic' plants. All experiments were conducted three times with at least three replicates in each assay. The results are shown as the averages $\pm$ standard deviation.

P. carotovorum subsp. *carotovorum* strain PccS1 (Cai et al. 2009). The promoterless kanamycin resistance gene was randomly inserted into wild-type PccS1, and a library comprising approximately 6,000 mutants was generated. A total of 500 insertion mutants displayed Km^r in the presence of plant extract but only one, PM86, showed

attenuated virulence on yellow-flowered calla (*Z. elliotiana*), calla lily (*Z. aethiopica*), and Chinese cabbage (*Brassica rapa* subsp. *pekinensis*) compared with the wild-type PccS1 (Supplementary Fig. S1). The other insertion mutants with plant extract-dependent Km^r caused wild-type virulence levels in host tissue.

***rplY* was identified at the site of the transposon insertion in PM86.** DNA from the positive transformants on LA containing Cm was amplified by a PCR assay using the transposon tagging primers Km-1 and 137-1. The products were sequenced and analyzed by BLASTn of the NCBI database, and the gene at the site of transposon insertion in PM86 was identified as *rplY*. *rplY* is 285 bp in length and encodes 95 amino acid residues of ribosomal protein L25 (RplY). RplY in PccS1 is identical to that in *P. aroidearum* strain PC1 isolated from monocotyledonous plant (Nabhan et al. 2013), which is conserved and shares around 93 to 94% similarity with other *Pectobacterium* spp. strains isolated from dicotyledonous plants, including *P. carotovorum* PCC21 and WPP14, *P. brasiliense* PBR1692, *P. wasabiae* WPP163, SCC3193 and 3304, and *P. atrosepticum* SCRI1043. When compared with other Enterobacteriaceae and Vibrionaceae species such as *D. dadantii* strain Ech586, *E. coli* strain MG1655, and *Vibrio cholera* strain V51, the RplY protein in PccS1 also shares 77, 70, and 61% identity, respectively (Fig. 1).

Plant extract induces bacterial growth in media. PM86 grew at wild-type levels in rich media (LB), and no difference showed in the growth of PM86 or the wild-type PccS1 in LB with and without plant extract (Supplementary Fig. S2). When cultured in MM or MM + ZE, PM86 showed attenuated growth in both conditions before 20 h. However, when cultured for an additional 12 h, the OD₆₀₀ of PM86 in MM + ZE almost increased to wild-type levels at stationary phase. In contrast, the OD₆₀₀ value of PM86 in MM did not exceed 0.2 by 32 h (Fig. 2).

***rplY* contributes to *P. carotovorum* subsp. *carotovorum* virulence on plant leaves.** As the transposon used to mutate target gene *rplY* might also affect genes in the flanking regions of *rplY*, we constructed a gene-deletion mutant of PccS1 ($\Delta rplY$) using homologous recombination. We also constructed a complemented strain $\Delta rplY(rplY)$ and a mutant containing the empty vector $\Delta rplY(pBBR)$. The deletion in *rplY* delayed the growth of the mutant in MM or MM + ZE for 16 h during the exponential growth phase but there were no significant differences in growth between these strains grown in MM or MM + ZE for 32 h (Fig. 2). The virulence of the wild-type PccS1 and mutant strains was determined on leaves of host plants from three different genera/species, namely *Z. elliotiana*, *Z. aethiopica* and *B. rapa* subsp. *pekinensis*. Compared with wild-type PccS1, $\Delta rplY$ was significantly attenuated in its ability to macerate these host

Fig. 3. Tissue maceration and bacterial population in planta of *Pectobacterium carotovorum* subsp. *carotovorum* strains: PccS1 (wild-type strain); $\Delta rplY$ (gene deletion mutant); $\Delta rplY(rplY)$ ($\Delta rplY$ complemented with the fragment of *rplY*); and $\Delta rplY(pBBR)$ ($\Delta rplY$ complemented with the empty vector carrying plasmid pBBR). +ZE, cells suspended with 3% (wt/vol) leaf extract of *Zantedeschia elliotiana* plants. **A**, Ten microliters of diluted bacterial cells (OD₆₀₀=1.2) of the appropriate cultures suspended with sterilized water (or ZE) was inoculated in parallel onto the leaves of colored calla (*Z. elliotiana* 'Black Magic'), calla lily (*Z. aethiopica*), and Chinese cabbage (*Brassica rapa* subsp. *pekinensis*). Photographs were taken 16 h after inoculation. **B**, Relative rate of the maceration area caused by the mutant strains compared with that caused by the wild-type PccS1 on the three hosts. Maceration area caused by PccS1 suspended by sterilized water is set to 1. The asterisks indicate a significant difference in lesion size caused by inoculation with the mutant strains versus PccS1 suspended in sterilized water. **C**, Quantities of the cells recovered from the macerated tissue per gram of rotted leaves of *Z. elliotiana* 16 h after inoculation. The experiments were repeated three times with at least 5 internal replicates in each. The results are shown as averages $\pm$ standard deviation. **P* < 0.05; ***P* < 0.01; ****P* < 0.001, versus wild-type PccS1, Duncan's multiple range test.

plants, while the complemented strain $\Delta rplY(rplY)$ partially restored this macerating ability (Fig. 3A and B). However, $\Delta rplY$ showed no reduction in colonization in *Z. elliotiana* leaves compared with the wild-type (Fig. 3C).

Deletion mutant $\Delta rplY$ exhibits reduced PCWDE activities and motility but has no effect on biofilm formation. The PCWDEs activities of the wild-type PccS1, $\Delta rplY$, $\Delta rplY(rplY)$, and $\Delta rplY(pBBR)$ strains were determined at 48 h after the cells were spotted onto assay plates. The PCWDEs activities were all significantly attenuated when gene *rplY* was deleted, and reduced activities were partially restored in the complemented strain $\Delta rplY(rplY)$ (Fig. 4A to C and E).

The results of qRT-PCR demonstrated that the expression of the genes encoding protease (*prtW*) and pectate lyase (*pel6*) in $\Delta rplY$ were significantly reduced at the transcriptional level. However, the deletion mutant exhibited a nonsignificant decrease in *celC* (cellulase) expression compared with the wild-type PccS1 (Fig. 4F).

The deletion mutant $\Delta rplY$ also showed a reduction in swimming motility on 0.3% 2YT agar plates and produced a sediment more quickly when cultured in tubes (Fig. 4D and G). qRT-PCR analysis showed that the transcript expression level of the flagellin gene (*fliC*) was significantly decreased in $\Delta rplY$ (Fig. 4F). Although FliC plays a crucial role in bacterial movement (Roessler and Muller 2002; Wang et al. 2007), transmission electron microscopy revealed that the flagella of

$\Delta rplY$ appeared similar in structure to those of the wild-type strain (data not shown). The result of crystal violet staining showed that deletion of *rplY* had no effect on biofilm formation by PccS1 (Fig. 4H and I).

Gene *rplY* is induced by plant extract. To demonstrate whether *rplY* was activated in the presence of plant extract, a chromosomal *rplY::lacZ* transcriptional fusion construct was designed. The promoter was significantly induced by the *Z. elliotiana* plant extract (Fig. 5A). PccS1 cells grown in MM + ZE showed higher expression levels of gene *rplY*. In the presence of plant extract, *rplY* was significantly upregulated at the 8-, 12-, and 16-h time-points, and particularly at 12 h (Fig. 5B). Moreover, the results of western blot analysis consolidated the finding that RplY expression was activated by plant extract. When cultured in MM + ZE, PccS1 cells produced more RplY protein than those in MM at 4, 12, 16, and 20 h (Fig. 5B).

DISCUSSION

RplY belongs to the ribosomal protein L25 family and has 92 to 95 amino acid residues (Fig. 1). RplY is bound independently to ribosomal 5S rRNA along with two other proteins RplE (L5) and RplR (L18), and forms a separate domain of the ribosome in *E. coli* (Lu and Steitz 2000). The protein sequence of RplY is highly conserved in strains of *Pectobacterium* isolated from monocotyledonous plants (Fig. 1), which might support the novel species *P. aroidearum* proposed

Fig. 4. Pleiotropic effects of *rplY* on *Pectobacterium carotovorum* subsp. *carotovorum* PccS1. From A to I, 1, wild-type strain PccS1; 2, gene deletion mutant $\Delta rplY$; 3, $\Delta rplY$ complemented with the fragment of *rplY*; and 4, strain of $\Delta rplY$ complemented with the empty vector carrying plasmid pBBR. **A, B, and C,** The plant cell wall-degrading enzymes (PCWDEs) activities were determined visually by the production of a clear halo around the colonies for protease, by double cream haloes around the colonies for pectate lyase, and by an off-white halo around the colonies for cellulase 48 h after the cells had been patched onto the assay plates. **D,** Swimming motility of the cells was represented by a halo on a clear background 24 h after inoculation on 0.3% agar plates. **E,** Bars indicate the halo areas on the different assay plates for the PCWDEs. Prt, Pel, and Cel are for protease, pectate lyase, and cellulase, respectively. **F,** Bars represent relative mRNA levels of the genes encoding the PCWDEs (*prtW*, *pel6*, and *celC*) and flagellin (*fliC*) in the wild-type PccS1 and the strains derived from PccS1 determined by quantitative real-time polymerase chain reaction. The housekeeping gene *recA* was used as an endogenous control. **G,** Sedimentation of the cells in glass tubes statically placed for 24 h at room temperature after it had been oscillated in 2YT at 28°C until the OD₆₀₀ reached 2.0. **H,** Biofilm development on the abiotic surface was revealed by the cells attached to the inner wall of the tubes and stained with crystal violet. **I,** Quantification of the biofilm measured by light absorbance at 575 nm of crystal violet eluted from the inner walls of the tubes. The experiments were repeated three times with at least 5 internal replicates. The results are shown as averages $\pm$ standard deviation. **P* < 0.05; ***P* < 0.01, versus the wild-type PccS1, Duncan's multiple range test.

by Nabhan in 2013 (Nabhan et al. 2013). RplY is essential for the production of mature 25S rRNA in *Saccharomyces cerevisiae* (Van Beekvelt et al. 2001), and is a surface-exposed protein that acts as an antigen recognizable by the host immune response in the animal pathogens *Neisseria meningitidis* and *Rickettsia heilongjiangensis* (Mendum et al. 2009; Qi et al. 2013).

Our results showed that *rplY* is important for the production of major PCWDEs in *P. carotovorum* subsp. *carotovorum*. Although expression of cellulase gene *celC* showed a nonsignificant decrease at the transcriptional level in $\Delta rplY$, it exhibited significantly attenuated cellulase activity on plates, which may be as a result of reduced *celC* expression at the translational level. Although we have no information on RplY in ribosomal assembly in *P. carotovorum* subsp. *carotovorum*, in the model species *E. coli* and *S. cerevisiae*, RplY is one of the ribosomal proteins bound to 5S rRNA and 25S rRNA, respectively (Lu and Steitz 2000; Van Beekvelt C. A. et al. 2001), 5S rRNA is one of components of the large subunit of the ribosome in all organisms. The large subunit catalyzes peptide bond formation via the enzyme peptidyltransferase (Woolford and Baserga 2013), so we speculate that reduced *celC* expression at the translational level might result from an impaired ribosomal protein in the absence of RplY.

In previous studies, it was revealed that motility is an important determinant of pathogenesis in *P. carotovorum* subsp. *carotovorum* (Hossain et al. 2005). Our motility assays (swimming and sedimentation) also suggest that, due to the additional effects on PCWDEs, RplY has a pleiotropic effect on the virulence of *P. carotovorum* subsp. *carotovorum*. Another ribosomal protein S12 (RpsL) in *P. carotovorum* subsp. *carotovorum* was also found with pleiotropic effects on virulence (Barnard et al. 2010). Barnard carried out a screen for transposon-induced *P. carotovorum* subsp.

carotovorum mutants showing defects in the production of carbapenem (Car), and identified such mutants that were also affected in virulence and PCWDE production. The mutants did not appear to carry the transposon but rather were affected by a single base change in the *rpsL* locus, resulting in the substitution of the wild-type lysine by threonine or asparagine at codon 43 (Barnard et al. 2010).

The reduced growth of PM86 in MM in the absence of plant extract might be directly linked to its attenuated virulence on plant leaves. Therefore, the virulence assays with PM86 were kept under observation over 32 h. However, although PM86 grew as well as the wild-type in rich media (LB), and PM86 populations reached wild-type levels at 32 HAI in MM + ZE, the lesion area at 32 HAI in the plant assay did not increase above that seen at 16 HAI. These results suggest that the attenuated virulence of PM86 resulted from impairment of *rplY* rather than reduction in bacterial population growth. The results also suggest that *rplY* is important for *P. carotovorum* subsp. *carotovorum* virulence.

The virulence of wild-type PccS1 increased on all three host plants when cells were suspended with 3% (wt/vol) plant extract from leaves of *Z. elliotiana* compared with sterilized water, although the differences were significant only on the leaves of *B. rapa* subsp. *pekinensis* (Fig. 3A and B). This is consistent with findings from previous studies in which extracts from the host plants of soft rot bacterial pathogens, such as carrot, potato, and celery, contain uncharacterized constituents that can induce pathogen virulence (Kersey et al. 2012). Since proteins larger than 5 kDa in the plant extract were removed, inducers in the extract are likely to be small molecules, as reviewed previously (Charkowski et al. 2012). Further studies are needed to characterize these putative inducers and to clarify the mechanisms underlying their effects on bacteria.

By recruiting compounds of plant extract as signals, pathogens modify the expression of genes to overcome host plant resistance (Kersey et al. 2012). In the present study, a total of 500 PccS1 mutants exhibited plant-extract-dependent kanamycin resistance. However, only *rplY* could be induced in the presence of *Z. elliotiana* extract, and PccS1 displayed attenuated virulence when *rplY* was impaired by insertion of the *mariner* transposon. Populations of the insertion mutant, PM86, increased to higher levels in MM + ZE than in MM after 20 h, suggesting that the plant extract might have nutrient effects on bacterial growth. However, growth of wild-type PccS1, $\Delta rplY$ and $\Delta rplY(rplY)$ increased slightly but not significantly in MM + ZE compared with MM (Fig. 2). Hence, we infer that the role of plant extract in bacterial growth is not as a nutrient source but rather acts as an inducer. This was further supported by the results of the *rplY::lacZ* transcriptional fusion report system and expression of *rplY* at transcriptional and translational levels in the wild-type PccS1 in the present of plant extract (Fig. 5).

Over the past several decades, proteomics, transcriptomics, and genomics studies have facilitated our understanding of bacteria-plant interactions (Bell et al. 2004; Judson and Mekalanos 2000; Mattinen et al. 2007; Nykyri et al. 2012; Toth et al. 2006). However, some crucial proteins are expressed at low levels that are undetectable by current approaches. In the present study, we showed that RplY was mainly expressed at low levels when *P. carotovorum* subsp. *carotovorum* strain PccS1 was grown in MM for 20 h. Interestingly, this protein was produced at a higher level in MM + ZE. RplY was not detected in the profiles of two-dimensional electrophoresis (2-DE) when we analyzed differentially expressed proteins in *in vivo* and *in vitro* interactions between PccS1 and *Z. elliotiana* (Wang et al. 2016).

In summary, we characterized gene *rplY* at the site of a random insertion by the *mariner* transposon, which was induced by plant extract and required for full virulence of *P. carotovorum* subsp. *carotovorum* strain PccS1. Our results suggest that transposon screening for potential virulence-related genes represents a complementary approach to omics studies, especially for proteins that are expressed at low levels during bacteria-plant interactions.

Fig. 5. Expression of *rplY* and RplY in *Pectobacterium carotovorum* subsp. *carotovorum* strain PccS1 induced by plant extract. **A**, The cells of PccS1 containing the *rplY::lacZ* transcriptional fusion were grown to an OD₆₀₀ of 2.0 in minimal medium (MM) and MM supplemented with 3% (wt/vol) plant extract of *Zantedeschia elliotiana* (MM + ZE). Cells were harvested and the β -galactosidase activities were determined. Bars depict the values of β -galactosidase conferred by the transcriptional *rplY::lacZ* fusion in the presence or absence of plant extract. **B**, Relative mRNA levels of *rplY* were determined by quantitative real-time polymerase chain reaction (qRT-PCR) in PccS1 cultured in minimal medium (MM) and MM supplemented with 3% ZE (wt/vol) for 4, 8, 12, 16, and 20 h. The mRNA levels of *rplY* in the cells cultured in MM were set to 1 to calculate the relative expression ratio. The expression of flag-tagged RplY in PccS1 cultured in MM and MM supplemented with 3% ZE (wt/vol) was analyzed by western blotting at the same time points as qRT-PCR. RNAP α was used as the loading control. The experiments were repeated three times with at least five internal replicates in each independent experiment. The results are shown as averages $\pm$ standard deviation. * P < 0.05; ** P < 0.01, versus the cells cultured in MM, Duncan's multiple range test.

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