Type VI Secretion Systems of *Erwinia amylovora* Contribute to Bacterial Competition, Virulence, and Exopolysaccharide Production

Yanli Tian, Yuqiang Zhao, Linye Shi, Zhongli Cui, Baishi Hu, and Youfu Zhao

First, third, and fifth authors: College of Plant Protection and Key Laboratory of Integrated Management of Crop Diseases and Pests, Ministry of Education, Nanjing Agricultural University, Nanjing 210095, China; second author: Institute of Botany, Jiangsu Province and the Chinese Academy of Sciences (Nanjing Botanical Garden Mem. Sun Yat-Sen), Nanjing 210014, China; fourth author: College of Life Sciences and Key Laboratory of Agricultural Environmental Microbiology, Ministry of Agriculture, Nanjing Agricultural University, Nanjing 210095, China; and sixth author: Department of Crop Sciences, University of Illinois at Urbana-Champaign, Urbana. Accepted for publication 10 February 2017.

ABSTRACT

The type VI secretion system (T6SS) plays a major role in mediating interbacterial competition and might contribute to virulence in plant pathogenic bacteria. However, the role of T6SS in *Erwinia amylovora* remains unknown. In this study, 33 deletion mutants within three T6SS clusters were generated in *E. amylovora* strain NCPB1665. Our results showed that all 33 mutants displayed reduced antibacterial activities against *Escherichia coli* as compared with that of the wild-type (WT) strain, indicating that *Erwinia amylovora* T6SS are functional. Of the 33 mutants, 19 exhibited reduced

virulence on immature pear fruit as compared with that of the WT strain. Among them, 6, 1, and 12 genes belonged to T6SS-1, T6SS-2, and T6SS-3 clusters, respectively. Interestingly, these 19 mutants also produced less amylovoran or levan or both. These findings suggest that *E. amylovora* T6SS play a role in bacterial competition and virulence possibly by influencing exopolysaccharide production.

Additional keywords: protein secretion system.

Fire blight, caused by the bacterium *Erwinia amylovora*, is a destructive bacterial disease of apple and pear as well as other rosaceous plants. Since the first reports of fire blight in the Hudson Valley of New York, the pathogen has continued to spread around the world and has now reached Central and East Asia, the origin of apple and pear germplasms (Jock et al. 2002; Myung et al. 2016; van der Zwet 2006; Vanneste 2000). The quarantine status of *E. amylovora* in many countries leads to further economic losses due to strict international trade regulations (Calvin and Krissoff 1998).

Gram-negative bacterial pathogens utilize at least six protein secretion systems to secrete effector proteins (Christie et al. 2005; Cornelis 2006; Segal et al. 2005). Among them, type VI secretion system (T6SS) is a unique system in proteobacteria (Bingle et al. 2008). The typical T6SS loci normally consists of 15 to 25 genes that can be divided into secreted proteins, such as hemolysin coregulated protein (Hcp) and valine-glycine repeat protein (VgrG), and non-secreted core apparatus or transport proteins, including a ClpV family of an AAA+ ATPase (Leung et al. 2011). A conserved 13 essential genes are shared by T6SS clusters (Boyer et al. 2009). It has been suggested that the T6SS injection machine appears as an inverted phage tail on the surface of the bacterial cell (Records 2011). VgrG proteins have a trimeric phage tail spike-like structure similar to the T4 bacteriophage gp27 and gp5 complex (Pukatzki et al. 2009), whereas Hcp from *Pseudomonas aeruginosa* forms hexameric rings that stack to form the extracellular and periplasmic tubule (Pukatzki et al. 2007). Small proline-alanine-alanine-arginine repeat proteins (PAAR proteins), or PAAR domains within larger proteins, interact

with VgrG to form a final sharp tip on the spike (Shneider et al. 2013). In *Vibrio cholera* (Records 2011), VipA and VipB form a complex of tubular, cogwheel-like structures. In both *V. cholerae* and *P. aeruginosa*, Hcp and VgrG proteins have been proposed to act as effectors associated with cytotoxicity (Mougous et al. 2006; Pukatzki et al. 2006). In addition, it has been reported that mutation of *vca0107* (*tssB*), *vca0108* (*tssC*), *vca0111* (*tssG*), *vca0114* (*tssK*), and *vca0120* (*tssM*) in *V. cholera* did not affect Hcp expression but Hcp secretion was abolished in those mutants (Bönemann et al. 2009; Ishikawa et al. 2012; Zheng et al. 2011). Silverman et al. (2011) showed that T6SS activity was negatively regulated by *PA0076* (*impM*) in *P. aeruginosa*.

T6SS gene clusters have been identified in at least 100 bacterial genomes (Records 2011). Among them, approximately one-third contain multiple T6SS, ranging from two to six (Boyer et al. 2009). Among the sequenced *E. amylovora* genomes, three T6SS gene clusters were identified (De Maayer et al. 2011; Mann et al. 2013; Smits et al. 2010) (Fig. 1). In *E. amylovora* strain CFBP 1430, T6SS-1 (Eamy_3028–Eamy_3000), T6SS-2 (Eamy_1623–Eamy_1620), and T6SS-3 (Eamy_3228–Eamy_3201) contain 27, 4, and 31 genes, respectively (De Maayer et al. 2011). Both T6SS-1 and T6SS-3 clusters encode approximately 15 core T6SS genes. Among them, at least three encode effector proteins (Hcp and VgrG) (De Maayer et al. 2011; Smits et al. 2010). Comparative genomic analysis revealed that variation between strains of *E. amylovora* is primarily found within the nonconserved *hcp* and *vgrG* islands of T6SS-1 regions II and IV and T6SS-3 region IV (De Maayer et al. 2011; Sarris et al. 2012). The T6SS-3 cluster is identified only in *E. amylovora* but is absent in other related *Erwinia* spp. (Mann et al. 2013).

Previous studies also suggested that T6SS may be involved in virulence of various animal and human pathogens, including *V. cholerae*, *P. aeruginosa*, *Edwardsiella tarda*, *Salmonella enteric* serovar *gallinarum*, and avian pathogenic *Escherichia coli* (Aschtgen et al. 2010; Blondel et al. 2010; Filloux et al. 2008; Hsu et al. 2009; Zheng and Leung 2007), as well as plant pathogens, including *Agrobacterium tumefaciens*, *Pectobacterium atrosepticum*, *Pseudomonas syringae*, *Pantoea ananatis*, *Ralstonia solanacearum*, *Rhizobium*

Corresponding authors: B. Hu; E-mail address: hbs@njau.edu.cn; and Y. Zhao; E-mail address: zhao888@illinois.edu

Y. Tian and Y. Zhao contributed equally to this research.

*The e-Xtra logo stands for “electronic extra” and indicates that one supplementary figure and three supplementary tables are published online.

leguminosum, and *Acidovorax citrulli* (Bingle et al. 2008; Bröms et al. 2013; Mattinen et al. 2007; Records and Gross 2010; Shyntum et al. 2015; Tian et al. 2015; Wu et al. 2008;). However, the role of T6SS in *Erwinia amylovora* remains unknown.

The objective of this study was to determine the role of *E. amylovora* T6SS in virulence and bacterial competition. Our results demonstrated that T6SS are involved in bacterial competition and played a role in virulence possibly by influencing exopolysaccharide (EPS) production.

MATERIALS AND METHODS

Bacterial stains and culture media. Bacterial strains and plasmids used in this study are listed in Table 1. The Luria-Bertani (LB) medium is used routinely for culturing *E. amylovora*. When necessary, the following antibiotics were added to the medium: gentamicin at 50 µg/ml and rifampicin at 100 µg/ml. Amylovoran production was determined by growing bacteria in 3 g of KH₂PO₄, 7 g of K₂HPO₄, 1 g of (NH₄)₂SO₄, 2 ml of glycerol, 0.5 g of citric acid, and 0.03 g of MgSO₄ (MBMA medium) plus 1% sorbitol (Bellemann et al. 1994; Zhao et al. 2009).

DNA manipulation and sequence analysis. Deletion mutants of T6SS core genes were generated by the *sacB*-based allele replacement method, as described previously (Zou et al. 2011). Deletion mutants were confirmed by polymerase chain reaction (PCR) amplification using primers flanking the genes of interest. Primer sequences used for mutant construction and confirmation are listed in Supplementary Table S1. The resultant constructs were introduced into *Escherichia coli* S17-1(λpir) for use in biparental mating with *Erwinia amylovora* strain NCPPB1665. For complementation, the coding regions of these genes were amplified by PCR using the primers listed in Supplementary Table S1 and cloned into the expression vector pBBR1MCS-5. The resulting constructs were transferred into the corresponding mutants by biparental mating.

Antibacterial competition assays. Antibacterial competition assays were conducted as described previously (Hachani et al. 2013; McNally et al. 2012). In brief, an *Escherichia coli* recipient cell was made by transforming *E. coli* DH5α cells with a plasmid pMD19-T allowing the α-complementation of the *lacZ* gene. The *Erwinia amylovora* attacker strain and the *Escherichia coli* DH5α (*lacZ*) target strain (both at an optical density at 600 nm [OD₆₀₀] = 0.5) were mixed at a ratio of 1:1. The “no *Erwinia amylovora*” control mixture contained sterile LB and *Escherichia coli* DH5α (*lacZ*) at a 1:1 ratio. For the quantification of the competition assay, 20 µl of this mixture was spotted onto solid LB medium for 5 h at 28°C. The CFU of surviving *E. coli* DH5α (*lacZ*) was measured by a serial dilution and selective growth on LB agar plates containing

rifampicin at 100 µg/ml (for *Erwinia amylovora*) and ampicillin at 100 µg/ml (for *Escherichia coli*), respectively. The experiment was repeated three times.

Virulence assay on immature pear fruit. Virulence tests on immature pear fruit (‘Cuiguan’) were performed as described previously (McNally et al. 2012; Wang et al. 2012). Briefly, bacterial cells were grown overnight in LB broth, harvested by centrifugation, and washed with double-distilled water (ddH₂O) three times. After the final wash, the pellet was resuspended in ddH₂O (OD₆₀₀ = 0.1 and then diluted 100-fold). Immature pear fruit were surface sterilized and pricked with a sterile needle. The wounded fruit were inoculated with 2 µl of cell suspensions and incubated at 100% relative humidity at 28°C. Symptoms were recorded at 8 days postinoculation. For each bacterial strain, three fruit were inoculated. Virulence was determined quantitatively via the percentage necrosis, calculated as the surface area of pear relative to the surface area of necrotic and water-soaked tissue. The experiment was repeated three times with similar results.

Cetylpyrimidinium chloride assay for determining amylovoran concentration. The amylovoran concentration in supernatants of bacterial cultures was quantitatively determined by a turbidity assay with cetylpyrimidinium chloride (CPC), as previously described (Bellemann et al. 1994; Wang et al. 2012; Zhao et al. 2009). Briefly, bacterial cells were grown overnight in LB broth, harvested by centrifugation, and washed with ddH₂O three times. After the final wash, the pellet was resuspended in 200 µl of ddH₂O. In total, 100 µl of bacterial suspension was inoculated into 10 ml of MBMA medium with 1% sorbitol. Bacterial cells (1 ml) were pelleted 2 days after inoculation at 28°C with shaking. Following centrifugation, 50 µl of CPC at 50 mg/ml was added to 1 ml of supernatant. After 10 min of incubation at room temperature, amylovoran concentration was determined by measuring OD₆₀₀ turbidity. The final concentration of amylovoran production was normalized for a cell density of 1.0. For each strain tested, the experiment was repeated three times.

Levansucrase assays. The secreted levansucrase activity was measured according to Hildebrand et al. (2006). Briefly, bacteria were cultured overnight in LB broth, and bacterial cells were removed by centrifugation. In total, 500 µl of supernatant was mixed with 500 µl of 100 mM potassium phosphate buffer (pH 7.0), 2 M sucrose and 0.05% sodium azide and incubated at 37°C for 3 h. Turbidity was measured at 600 nm and normalized for a cell density of 1.0. For each strain tested, the experiment was repeated three times.

RNA isolation. For *ams* gene expression, bacterial strains were grown overnight in LB medium, diluted in 5 ml of MBMA medium at an OD₆₀₀ of 0.005, and incubated for 18 h of growth in MBMA

Fig. 1. Arrangement of *Erwinia amylovora* CFBP 1430 type VI secretion system (T6SS) gene clusters indicating the relative position of gene homologs. **A**, T6SS gene cluster 1; **B**, T6SS gene cluster 2; and **C**, T6SS gene cluster 3.

medium at 28°C. For *lsc* gene expression, *Erwinia amylovora* strains were cultured overnight in LB broth as described above. RNA Protect Reagent (2 ml) (Qiagen) was added to 1 ml of bacterial culture (at OD₆₀₀ of approximately 0.5 to 0.8) to stabilize RNA. Cells were harvested by centrifugation for 10 min at 4,000 × *g* and RNA was extracted using Qiagen Bacterial RNA Protect Mini Kit as recommended by the manufacturer (Qiagen). On-column DNA digestion was performed using Qiagen DNase. RNA integrity was confirmed by electrophoresis on 1.2% agarose gels.

Quantitative 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). 16S ribosomal RNA (rRNA) was used as an endogenous control. Primer sequences are listed in Supplementary Table S2. Primer pairs 16SF-16SR, *amsB*-*amsBR*, *amsD*-*amsDR*, *amsE*-*amsER*, *amsG*-*amsGR*, *amsJ*-*amsJR*, and *lscF*-*lscR* were used to detect expression of 16S rRNA, *amsB*, *amsD*, *amsE*, *amsG*, *amsJ*, and *lsc* genes, respectively. Then, 2 µg of each RNA sample

TABLE 1. Bacterial strains and plasmids used in this study

Bacteria or plasmids	Relevant characteristics ^z	Source or reference
<i>Erwinia amylovora</i>		
NCPBP1665	Wild-type, Rif ^R	Lab collection
Δ <i>vgrG</i> -2	<i>vgrG</i> -2 knockout mutant	This study
Δ <i>vgrG</i> -1	<i>vgrG</i> -1 knockout mutant	This study
Δ <i>clpV</i> -1	<i>clpV</i> -1 knockout mutant	This study
Δ <i>tssG</i> -1	<i>tssG</i> -1 knockout mutant	This study
Δ <i>tssF</i> -1	<i>tssF</i> -1 knockout mutant	This study
Δ <i>tssE</i> -1	<i>tssE</i> -1 knockout mutant	This study
Δ <i>tagH</i> -1	<i>tagH</i> -1 knockout mutant	This study
Δ <i>hcp</i> -1	<i>hcp</i> -1 knockout mutant	This study
Δ <i>tssC</i> -1	<i>tssC</i> -1 knockout mutant	This study
Δ <i>tssB</i> -1	<i>tssB</i> -1 knockout mutant	This study
Δ <i>tssA</i> -1	<i>tssA</i> -1 knockout mutant	This study
Δ <i>tssM</i> -1	<i>tssM</i> -1 knockout mutant	This study
Δ <i>tssL</i> -1	<i>tssL</i> -1 knockout mutant	This study
Δ <i>tssK</i> -1	<i>tssK</i> -1 knockout mutant	This study
Δ <i>tssJ</i> -1	<i>tssJ</i> -1 knockout mutant	This study
Δ <i>tagH</i> -2	<i>tagH</i> -2 knockout mutant	This study
Δ <i>tssM</i> -2	<i>tssM</i> -2 knockout mutant	This study
Δ <i>tssL</i> -2	<i>tssL</i> -2 knockout mutant	This study
Δ <i>tssA</i> -3	<i>tssA</i> -3 knockout mutant	This study
Δ <i>tssE</i> -2	<i>tssE</i> -2 knockout mutant	This study
Δ <i>vgrG</i> -4	<i>vgrG</i> -4 knockout mutant	This study
Δ <i>tssJ</i> -2	<i>tssJ</i> -2 knockout mutant	This study
Δ <i>tssG</i> -2	<i>tssG</i> -2 knockout mutant	This study
Δ <i>tssF</i> -2	<i>tssF</i> -2 knockout mutant	This study
Δ <i>tssA</i> -2	<i>tssA</i> -2 knockout mutant	This study
Δ <i>tssM</i> -3	<i>tssM</i> -3 knockout mutant	This study
Δ <i>vgrG</i> -3	<i>vgrG</i> -3 knockout mutant	This study
Δ <i>clpV</i> -2	<i>clpV</i> -2 knockout mutant	This study
Δ <i>hcp</i> -2	<i>hcp</i> -2 knockout mutant	This study
Δ <i>tssL</i> -3	<i>tssL</i> -3 knockout mutant	This study
Δ <i>tssK</i> -2	<i>tssK</i> -2 knockout mutant	This study
Δ <i>tssC</i> -2	<i>tssC</i> -2 knockout mutant	This study
Δ <i>tssB</i> -2	<i>tssB</i> -2 knockout mutant	This study
<i>Escherichia coli</i>		
DH5α(λpir)	Φ80 <i>lacZ</i> M15, Δ(<i>lacZYA-argF</i>) U169. <i>recA1</i> , <i>endA1</i> . <i>thi-1</i>	TaKaRa
S17-1(λpir)	<i>Apir pro hsdR</i> , <i>recA</i>	Simon et al. 1983
Plasmids		
pMD19-T	“TA” Cloning vector	TaKaRa
pEX18GM	Suicide vector with a <i>sacB</i> gene, Gm ^R	Hoang et al. 1998
pBBR1MCS-5	Broad-host-range cloning vector, Gm ^R	Kovach et al. 1995
pVgrG2	Gm ^R , a 2,764-bp DNA fragment containing <i>vgrG</i> -2 gene and its promoter in pBBR1MCS-5	This study
pVgrG1	Gm ^R , a 2,503-bp DNA fragment containing <i>vgrG</i> -1 gene and its promoter in pBBR1MCS-5	This study
pTssG1	Gm ^R , a 1,484-bp DNA fragment containing <i>tssG</i> -1 gene and its promoter in pBBR1MCS-5	This study
pTagH1	Gm ^R , a 2,085-bp DNA fragment containing <i>tagH</i> -1 gene and its promoter in pBBR1MCS-5	This study
pTssM1	Gm ^R , a 4,133-bp DNA fragment containing <i>tssM</i> -1 gene and its promoter in pBBR1MCS-5	This study
pTssK1	Gm ^R , a 1,735-bp DNA fragment containing <i>tssK</i> -1 gene and its promoter in pBBR1MCS-5	This study
pTagH2	Gm ^R , a 1,585-bp DNA fragment containing <i>tagH</i> -2 gene and its promoter in pBBR1MCS-5	This study
pTssA3	Gm ^R , a 1,916-bp DNA fragment containing <i>tssA</i> -3 gene and its promoter in pBBR1MCS-5	This study
pTssE2	Gm ^R , a 705-bp DNA fragment containing <i>tssE</i> -2 gene and its promoter in pBBR1MCS-5	This study
pTssJ2	Gm ^R , a 828-bp DNA fragment containing <i>tssJ</i> -2 gene and its promoter in pBBR1MCS-5	This study
pTssG2	Gm ^R , a 1,634-bp DNA fragment containing <i>tssG</i> -2 gene and its promoter in pBBR1MCS-5	This study
pTssF2	Gm ^R , a 1,974-bp DNA fragment containing <i>tssF</i> -2 gene and its promoter in pBBR1MCS-5	This study
pTssA2	Gm ^R , a 1,974-bp DNA fragment containing <i>tssA</i> -2 gene and its promoter in pBBR1MCS-5	This study
pTssM3	Gm ^R , a 3,783-bp DNA fragment containing <i>tssM</i> -3 gene and its promoter in pBBR1MCS-5	This study
pClpV2	Gm ^R , a 1,783-bp DNA fragment containing <i>clpV</i> -2 gene and its promoter in pBBR1MCS-5	This study
pTssL3	Gm ^R , a 939-bp DNA fragment containing <i>tssL</i> -3 gene and its promoter in pBBR1MCS-5	This study
pTssK2	Gm ^R , a 1,950-bp DNA fragment containing <i>tssK</i> -2 gene and its promoter in pBBR1MCS-5	This study
pTssC2	Gm ^R , a 2,078-bp DNA fragment containing <i>tssC</i> -2 gene and its promoter in pBBR1MCS-5	This study
pTssB2	Gm ^R , a 1,064-bp DNA fragment containing <i>tssB</i> -2 gene and its promoter in pBBR1MCS-5	This study

^z Gm^R and Rif^R indicate resistance to gentamicin and rifampicin, respectively.

was used to synthesize cDNA with a cDNA synthesis kit (TaKaRa). Quantitative real-time PCR amplifications were carried out at 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, and a final dissociation curve analysis step from 65 to 95°C. The experiment was repeated three times. The transcriptional levels of *amsB*, *amsD*, *amsE*, *amsG*, *amsJ*, and *lsc* in the wild-type (WT) and mutant strains was assessed and compared.

Data analysis. All analyses were conducted using SPSS 14.0 (SPSS Inc.). The one-way student's *t* test (*P* = 0.05) was used to determine significant differences in disease severity, pathogenicity-related phenotypes, and gene expressions among WT and mutant strains of *E. amylovora*.

RESULTS

T6SS of *E. amylovora* play a role in antibacterial activities.

To test the contribution of T6SS to the antibacterial properties of *Erwinia amylovora*, we examined the abilities of 33 T6SS mutants in competitively killing *Escherichia coli* DH5 α on agar plates. Survival of *Escherichia coli* was lower when coinoculated with the *Erwinia amylovora* WT, whereas survival of *Escherichia coli* was greatly increased when coinoculated with all 33 mutants (Fig. 2). The survival of *Escherichia coli* was decreased in complementation strains (Supplementary Table S3). These results indicated that T6SS in *Erwinia amylovora* play a role in bacterial competition, suggesting that T6SS in *E. amylovora* are functional.

T6SS of *E. amylovora* contribute to virulence. Virulence tests of the 33 T6SS mutants on immature pear fruit showed that 19 mutants (Δ *tssA*-2, Δ *tssA*-3, Δ *tssB*-2, Δ *tssC*-2, Δ *tssE*-2, Δ *tssF*-2, Δ *tssG*-1, Δ *tssG*-2, Δ *tssJ*-2, Δ *tssK*-1, Δ *tssK*-2, Δ *tssL*-3, Δ *tssM*-1, Δ *tssM*-3, Δ *vgrG*-1, Δ *vgrG*-2, Δ *clpV*-2, Δ *tagH*-1, and Δ *tagH*-2) were either nonpathogenic or their virulence was reduced on immature pear fruit as compared with that of the WT strain (Fig. 3). Among them, 6, 1, and 12 belong to the T6SS-1, T6SS-2, and T6SS-3 clusters, respectively. Complementation of these mutants partially or completely recovered their virulence to the level of the WT (Fig. 3). We have compared the growth of the WT and these mutants (Supplementary Fig. S1), and found that the mutants were not impaired in growth. The other 14 mutants (Δ *tssA*-1, Δ *tssB*-1, Δ *tssC*-1, Δ *tssE*-1, Δ *tssF*-1, Δ *tssJ*-1, Δ *tssL*-1, Δ *tssL*-2, Δ *tssM*-2, Δ *clpV*-1, Δ *hcp*-1, Δ *hcp*-2, Δ *vgrG*-3, and Δ *vgrG*-4) were as pathogenic as the WT strain (data not shown). These results indicated that T6SS systems of *E. amylovora* contribute to virulence.

T6SS affect amylovoran production. Phenotypes of the 33 deletion mutant strains were further evaluated by measuring amylovoran

biosynthesis in vitro. Three groups of mutants exhibiting varying levels of amylovoran production in vitro were identified (Table 2). For group I, four mutants (Δ *tssA*-1, Δ *tssB*-1, Δ *tssC*-1, and Δ *hcp*-1) showed approximately twofold increase of amylovoran production as compared with that of the WT (Table 2). Consistent with amylovoran production, amylovoran biosynthetic genes, including *amsB*, *amsD*, *amsE*, *amsG*, and *amsJ*, were upregulated by two- to threefold in these four mutant strains (Table 3). For group 2, 16 mutants showed a 1.3- to 5-fold reduction in amylovoran production as compared with that of the WT; these included Δ *tssA*-2, Δ *tssA*-3, Δ *tssB*-2, Δ *tssC*-2, Δ *tssE*-2, Δ *tssF*-2, Δ *tssG*-1, Δ *tssG*-2, Δ *tssJ*-2, Δ *tssK*-1, Δ *tssK*-2, Δ *tssL*-3, Δ *tssM*-3, Δ *clpV*-2, Δ *vgrG*-1, and Δ *vgrG*-2 (Table 2). Expression of *amsB*, *amsD*, *amsE*, *amsG*, and *amsJ* genes were also downregulated by two- to fivefold in these 16 mutant strains (Table 3). Amylovoran production was complemented in group 1 and 2 mutant strains. Group 3 mutants (13 mutants) did not show much difference in amylovoran production when compared with that of the WT; these included Δ *tssE*-1, Δ *tssF*-1, Δ *tssJ*-1, Δ *tssL*-1, Δ *tssL*-2, Δ *tssM*-1, Δ *tssM*-2, Δ *hcp*-2, Δ *clpV*-1, Δ *tagH*-1, Δ *tagH*-2, Δ *vgrG*-3, and Δ *vgrG*-4 (Table 2). These results indicated that T6SS influence amylovoran production in *E. amylovora*.

T6SS influence levan production. Levan production was also determined in WT and 33 mutants. Fourteen mutants had a two- to fourfold reduction in levan production as compared with that of the WT strain; these included Δ *tssK*-1, Δ *tssM*-1, Δ *tssA*-1, Δ *hcp*-1, Δ *tagH*-1, Δ *tssE*-1, Δ *tssF*-1, Δ *tssG*-1, Δ *tagH*-2, Δ *tssK*-2, Δ *tssM*-3, Δ *tssA*-2, Δ *tssF*-2, and Δ *tssJ*-2 (Table 2). Levan production was complemented in these mutants. Similarly, expression of the *lsc* gene was also decreased two- to eightfold in these 14 mutants as compared with that of the WT strain (Table 3). These results indicated that T6SS positively affect levan production in *E. amylovora*.

DISCUSSION

In the sequenced genomes of *E. amylovora* strains, three T6SS gene clusters were identified (Mann et al. 2013; Smits et al. 2010). Comparison with other related *Erwinia* spp. on pome fruit trees indicated that T6SS-1 and T6SS-2 are shared among them, whereas T6SS-3 is present only in *E. amylovora* strains (Mann et al. 2013; Smits et al. 2010). Previous studies have shown that T6SS mediates interbacterial interactions (Ma et al. 2014; Schwarz et al. 2010; Shyntum et al. 2015), virulence (Pukatzki et al. 2006; Suarez et al. 2008; Zheng and Leung 2007), biofilm formation (Aschtgen et al. 2010), quorum sensing, and stress responses (Weber et al. 2009), thus enhancing their adaptation abilities to changing environmental

Fig. 2. Competition assay between *Erwinia amylovora* and *Escherichia coli*. Recovered *Escherichia coli* DH5 α after coinoculation with *Erwinia amylovora* wild type (WT) and mutants. “No *E. amylovora*” control mixture contained sterile Luria-Bertani medium and *Escherichia coli* DH5 α at a 1:1 ratio. Vertical bars represent standard errors of the means.

conditions (Weber et al. 2009). In this study, we characterized 33 deletion mutants of the T6SS core genes in *E. amylovora* strain NCPPB1665 and demonstrated that all three T6SS systems (T6SS-1, T6SS-2, and T6SS-3) were involved in bacterial competition, EPS production, and virulence in the fire blight pathogen. These results also suggest that T6SS might contribute to virulence, possibly by promoting amylovoran and levan biosynthesis.

EPS has been reported to play a key role in protecting bacteria against water and nutrient loss and to bypass the plant defense system (Denny 1995; Ordax et al. 2010). *E. amylovora* produces two types of EPS, levan and amylovoran (Ayers et al. 1979), where amylovoran is considered to be a pathogenicity factor and levan a virulence factor

(Gross et al. 1992; Sjulín and Beer 1978; Vrancken et al. 2013). Although T6SS have been known to play a role in virulence of various animal, human, and plant pathogens, the connection between T6SS and EPS has not been reported previously (Aschtgen et al. 2010; Blondel et al. 2010; Filloux et al. 2008; Hsu et al. 2009; Mattinen et al. 2007; Records and Gross 2010; Wu et al. 2008). In this study, among the 19 T6SS mutants with reduced virulence, 7 mutants produced less amylovoran and levan, 9 produced less amylovoran, and 3 produced less levan. These results suggest the possible link between virulence reductions in the T6SS mutants with EPS production.

Accumulation of amylovoran could lead to wilting symptoms by restriction of water movement through physical blockage of

Fig. 3. Virulence tests on immature pear fruit. **A**, Symptoms caused by the *Erwinia amylovora* wild type (WT), type VI secretion system (T6SS) mutants, and complementation strains on immature Cuiguan pear fruit. Virulence assays were performed using immature pear fruit by pricking with a needle and inoculating 2 μ l of pathogen suspension (optical density at 600 nm = 0.1, then diluted 100-fold, as described in Materials and Methods) onto the wounded tissue. The experiment was performed three times with similar results. Pictures were taken at 8 days postinoculation. **B**, Quantitative measurements of the percentage of necrotic areas. Vertical bars represent standard errors of the means. T6SS-1, -2, and -3 indicates genes from three T6SS gene clusters.

vascular elements in *E. amylovora* (Sjulin and Beer 1978). Genetic studies showed that amylovoran-deficient mutants are avirulent (Bellemann et al. 1994; Steinberger 1988) and the quantity of amylovoran produced by individual *E. amylovora* strains is correlated with the degree of virulence (Ayers et al. 1979). Genome-wide screening of two-component signal transduction systems (TCST) identified four groups of TCST as either negative or positive regulators of amylovoran biosynthesis in *E. amylovora*, including the RcsBCD, GacS/GacA, and EnvZ/OmpR systems (Zhao et al. 2009). Genetic screening also identified other global regulatory genes for amylovoran biosynthesis, including AmyR, Lon protease, c-di-GMP, small RNAs, and H-NS (Eastgate et al. 1995; Edmunds et al. 2013; Hildebrand et al. 2006; Wang et al. 2012; Zeng et al. 2013). These findings suggest that regulation of amylovoran biosynthesis is highly complex. In the current study, we provided evidence that EPS production may also be under the influence of the T6SS. Though we do not have a definite answer as to why, it is tempting to speculate that T6SS may provide *E. amylovora* some advantage to compete with other microorganisms in an environment with limited resources, or to protect *E. amylovora* from other microorganisms which may also utilize T6SS to attack by producing more amylovoran or levan.

T6SS has been well established under laboratory conditions for its role in bacterial competition (Dong et al. 2013; Hood et al. 2010); recent study also indicated that *P. ananatis* T6SS-1 plays an important role in pathogenesis on onion plants and in bacterial competition (Shyntum et al. 2015). We observed similar antibacterial

activity of T6SS during coculture on a solid agar surface, suggesting that *E. amylovora* acts through neighboring cells, as proposed for other bacteria (Dong et al. 2013; Ma et al. 2014). Because conserved T6SS effectors such as Tae and Tle are not present in the *E. amylovora* genome, it is still unclear whether T6SS deliver other effectors in natural communities or how T6SS in *E. amylovora* function in both pathogenesis and bacterial competition. Further investigation is warranted to demonstrate the molecular mechanisms.

Some genes with dual functions have been reported in previous studies (Matthysse et al. 1996; Zhang et al. 2002). For example, in addition to being involved in the attachment of *Agrobacterium tumefaciens* to plant cells (Matthysse et al. 1996), *attM* was identified to encode the nacylhomoserine lactone quorum-sensing signal molecule degradation enzyme (Zhang et al. 2002). A mutant of the *attM* gene showed a nonattachment phenotype and decreased virulence (Matthysse et al. 1996). Furthermore, Zhang et al. (2002) presented evidence that the enzyme encoded by *attM* inactivates 3OC8HSL quorum-sensing signals by hydrolysis of its homoserine lactone ring. We found that some T6SS core components influenced both EPS production and bacterial competition. Our results also indicate that all of the core genes contributed to bacterial competition. The T6SS core components form a complex transenvelope machinery which directly injects secreted effector proteins from the bacterial cytoplasm into a target cell (Boyer et al. 2009; Ho et al. 2014). It is easy to understand that all 33 core gene mutants displayed reduced antibacterial activities against *Escherichia coli* as compared

TABLE 2. Virulence and exopolysaccharide production of *Erwinia amylovora* mutants and the wild type (WT)^y

WT, mutant ^z	Virulence (% necrotic)	Amylovoran production (OD ₆₀₀)	Levan production (OD ₆₀₀)
WT	92 ± 2.2 a	1.794 ± 0.036 c	0.465 ± 0.039 a
T6SS-1			
ΔvgrG-2	38.3 ± 12.2 b	1.316 ± 0.091 de	0.403 ± 0.011 ab
ΔvgrG-1	40.7 ± 6.22 b	1.28 ± 0.02 e	0.407 ± 0.009 ab
ΔclpV-1	81.67 ± 5.556 a	1.8 ± 0.12 c	0.46 ± 0.02 a
ΔtssG-1	0 e	0.363 ± 0.038 g	0.266 ± 0.037 def
ΔtssF-1	71.67 ± 4.444 a	1.75 ± 0.04 c	0.258 ± 0.031 def
ΔtssE-1	76.67 ± 2.222 a	1.773 ± 0.071 c	0.083 ± 0.036 g
ΔtagH-1	0 e	1.763 ± 0.142 c	0.042 ± 0.039 g
Δhcp-1	73.33 ± 8.889 a	<i>3.713 ± 0.158</i> a	0.053 ± 0.031 g
ΔtssC-1	78.33 ± 5.556 a	<i>3.045 ± 0.122</i> b	0.369 ± 0.012 ab
ΔtssB-1	85 ± 6.667 a	<i>3.03 ± 0.173</i> b	0.372 ± 0.021 ab
ΔtssA-1	83.33 ± 8.889 a	<i>2.933 ± 0.038</i> b	0.253 ± 0.016 def
ΔtssM-1	23.33 ± 4.444 cd	1.817 ± 0.031 c	0.085 ± 0.034 g
ΔtssL-1	88.33 ± 2.222 a	1.8 ± 0.067 c	0.319 ± 0.026 ab
ΔtssK-1	1.667 ± 2.222 e	0.96 ± 0.053 f	0.209 ± 0.015 ef
ΔtssJ-1	78.33 ± 7.778 a	1.743 ± 0.058 c	0.42 ± 0.02 ab
T6SS-2			
ΔtagH-2	41.7 ± 5.56 b	1.713 ± 0.104 c	0.248 ± 0.009 def
ΔtssM-2	81.67 ± 8.889 a	1.747 ± 0.076 c	0.448 ± 0.019 a
ΔtssL-2	90 ± 13.33 a	1.79 ± 0.087 c	0.417 ± 0.045 a
T6SS-3			
ΔtssA-3	0 e	0.463 ± 0.051 g	0.448 ± 0.018 a
ΔtssE-2	0 e	0.477 ± 0.116 g	0.435 ± 0.011 a
ΔvgrG-4	90 ± 3.33 a	1.78 ± 0.047 c	0.46 ± 0.024 a
ΔtssJ-2	31.7 ± 5.56 c	0.753 ± 0.089 f	0.271 ± 0.025 def
ΔtssG-2	5 ± 3.33 e	0.407 ± 0.038 g	0.443 ± 0.022 a
ΔtssF-2	23.3 ± 8.89 cd	0.773 ± 0.051 f	0.149 ± 0.035 fg
ΔtssA-2	23.33 ± 4.444 cd	0.802 ± 0.038 f	0.282 ± 0.016 def
ΔtssM-3	1.67 ± 2.22 e	0.503 ± 0.038 g	0.208 ± 0.057 ef
ΔvgrG-3	90 ± 6.667 a	1.75 ± 0.047 c	0.447 ± 0.012 a
ΔclpV-2	6.67 ± 4.44 de	0.353 ± 0.069 g	0.423 ± 0.004 a
Δhcp-2	81.67 ± 7.778 a	1.733 ± 0.078 c	0.417 ± 0.011 ab
ΔtssL-3	15 ± 3.33 d	0.92 ± 0.1337 f	0.303 ± 0.012 ab
ΔtssK-2	18.3 ± 5.56 d	1.34 ± 0.167 de	0.071 ± 0.046 g
ΔtssC-2	0 e	0.83 ± 0.04 f	0.451 ± 0.02 a
ΔtssB-2	1.67 ± 2.22 e	0.45 ± 0.033 g	0.455 ± 0.021 a

^y Values within virulence, amylovoran production, and levan production followed by the same letter are not significantly different according to one-way Student's *t* test (*P* = 0.05). OD₆₀₀ = optical density at 600 nm. Numbers in bold indicate reduced compared with the WT and numbers in italics indicate increased compared with the WT.

^z T6SS = type VI secretion system.

TABLE 3. Expression ratio of amylovoran and levan biosynthesis genes in type VI secretion system (T6SS) mutant strains compared with the wild-type strain

Strain	Genes					
	<i>amsB</i> (EAMY_2249)	<i>amsD</i> (EAMY_2247)	<i>amsE</i> (EAMY_2246)	<i>amsG</i> (EAMY_2253)	<i>amsJ</i> (EAMY_2244)	<i>lsc</i> (EAMY_3695)
T6SS-1						
<i>ΔvgrG-2</i>	0.51 ± 0.07	0.50 ± 0.13	0.53 ± 0.18	0.57 ± 0.10	0.53 ± 0.12	1.07 ± 0.18
<i>ΔvgrG-1</i>	0.47 ± 0.16	0.52 ± 0.10	0.51 ± 0.14	0.43 ± 0.16	0.50 ± 0.11	1.20 ± 0.07
<i>ΔclpV-1</i>	1.04 ± 0.16	1.03 ± 0.17	1.03 ± 0.16	0.93 ± 0.18	0.87 ± 0.16	1.02 ± 0.18
<i>ΔtssG-1</i>	0.25 ± 0.09	0.21 ± 0.01	0.43 ± 0.07	0.40 ± 0.05	0.44 ± 0.08	0.52 ± 0.06
<i>ΔtssF-1</i>	1.03 ± 0.17	1.00 ± 0.08	1.07 ± 0.19	1.01 ± 0.14	0.89 ± 0.14	0.51 ± 0.07
<i>ΔtssE-1</i>	1.10 ± 0.06	1.05 ± 0.15	1.14 ± 0.04	1.16 ± 0.12	0.96 ± 0.16	0.12 ± 0.06
<i>ΔtagH-1</i>	1.07 ± 0.18	0.97 ± 0.11	0.80 ± 0.20	0.83 ± 0.04	0.92 ± 0.19	0.16 ± 0.08
<i>Δhcp-1</i>	3.63 ± 0.29	3.40 ± 0.27	3.77 ± 0.29	3.23 ± 0.51	3.50 ± 0.47	0.20 ± 0.05
<i>ΔtssC-1</i>	2.57 ± 0.42	3.40 ± 0.27	3.27 ± 0.32	3.03 ± 0.31	3.02 ± 0.33	1.01 ± 0.07
<i>ΔtssB-1</i>	2.53 ± 0.44	2.53 ± 0.58	2.41 ± 0.27	2.63 ± 0.42	2.73 ± 0.22	1.07 ± 0.06
<i>ΔtssA-1</i>	2.67 ± 0.44	2.37 ± 0.42	2.70 ± 0.33	2.67 ± 0.24	2.77 ± 0.18	0.54 ± 0.09
<i>ΔtssM-1</i>	0.83 ± 0.05	0.88 ± 0.14	1.09 ± 0.14	0.86 ± 0.08	0.83 ± 0.10	0.16 ± 0.04
<i>ΔtssL-1</i>	0.95 ± 0.17	1.10 ± 0.14	1.16 ± 0.12	0.84 ± 0.04	1.02 ± 0.12	1.07 ± 0.02
<i>ΔtssK-1</i>	0.42 ± 0.09	0.34 ± 0.04	0.40 ± 0.06	0.49 ± 0.06	0.52 ± 0.05	0.22 ± 0.07
<i>ΔtssJ-1</i>	1.19 ± 0.04	1.01 ± 0.09	0.90 ± 0.15	1.09 ± 0.16	1.09 ± 0.13	1.10 ± 0.15
T6SS-2						
<i>ΔtagH-2</i>	0.99 ± 0.09	1.10 ± 0.20	1.01 ± 0.15	0.91 ± 0.07	0.89 ± 0.02	0.62 ± 0.09
<i>ΔtssM-2</i>	1.01 ± 0.14	0.83 ± 0.05	0.89 ± 0.13	0.87 ± 0.08	1.10 ± 0.14	1.08 ± 0.13
<i>ΔtssL-2</i>	0.97 ± 0.16	0.95 ± 0.17	0.87 ± 0.16	0.88 ± 0.05	0.94 ± 0.12	1.14 ± 0.11
T6SS-3						
<i>ΔtssA-3</i>	0.28 ± 0.05	0.43 ± 0.09	0.45 ± 0.04	0.42 ± 0.02	0.53 ± 0.04	0.85 ± 0.10
<i>ΔtssE-2</i>	0.33 ± 0.02	0.31 ± 0.06	0.44 ± 0.05	0.44 ± 0.02	0.51 ± 0.02	0.88 ± 0.09
<i>ΔvgrG-4</i>	0.96 ± 0.16	1.10 ± 0.20	0.91 ± 0.14	0.97 ± 0.16	0.97 ± 0.12	1.03 ± 0.11
<i>ΔtssJ-2</i>	0.38 ± 0.10	0.28 ± 0.05	0.32 ± 0.05	0.41 ± 0.04	0.37 ± 0.08	0.50 ± 0.11
<i>ΔtssG-2</i>	0.22 ± 0.05	0.30 ± 0.06	0.34 ± 0.09	0.28 ± 0.05	0.37 ± 0.11	0.97 ± 0.16
<i>ΔtssF-2</i>	0.35 ± 0.09	0.30 ± 0.07	0.32 ± 0.05	0.39 ± 0.02	0.34 ± 0.05	1.07 ± 0.22
<i>ΔtssA-2</i>	0.38 ± 0.08	0.40 ± 0.07	0.37 ± 0.04	0.45 ± 0.06	0.35 ± 0.03	1.13 ± 0.24
<i>ΔtssM-3</i>	0.28 ± 0.05	0.35 ± 0.11	0.48 ± 0.10	0.36 ± 0.06	0.40 ± 0.11	1.03 ± 0.11
<i>ΔvgrG-3</i>	0.92 ± 0.19	1.21 ± 0.06	1.01 ± 0.20	0.98 ± 0.18	1.07 ± 0.18	0.87 ± 0.04
<i>ΔclpV-2</i>	0.28 ± 0.05	0.25 ± 0.05	0.40 ± 0.08	0.35 ± 0.04	0.38 ± 0.09	0.89 ± 0.06
<i>Δhcp-2</i>	1.10 ± 0.07	1.03 ± 0.16	0.87 ± 0.05	1.07 ± 0.12	1.14 ± 0.10	0.97 ± 0.16
<i>ΔtssL-3</i>	0.40 ± 0.06	0.35 ± 0.04	0.33 ± 0.04	0.46 ± 0.04	0.46 ± 0.06	1.02 ± 0.12
<i>ΔtssK-2</i>	0.47 ± 0.24	0.53 ± 0.42	0.50 ± 0.20	0.37 ± 0.29	0.43 ± 0.11	0.34 ± 0.14
<i>ΔtssC-2</i>	0.43 ± 0.11	0.41 ± 0.06	0.42 ± 0.10	0.31 ± 0.03	0.43 ± 0.08	0.93 ± 0.11
<i>ΔtssB-2</i>	0.30 ± 0.06	0.32 ± 0.06	0.40 ± 0.07	0.29 ± 0.06	0.44 ± 0.06	1.07 ± 0.18

with that of the WT strain; however, we still do not have a reasonable explanation as to why some genes but not the others are involved in EPS production. Future mechanism studies are needed to clarify this. In summary, we provided evidence that T6SS function in bacterial competition in *Erwinia amylovora* strain NCPPB1665. Our data also provided experimental evidence that T6SS play an important role in virulence, possibly by affecting EPS production in *E. amylovora*. Future works should focus on determining the molecular mechanisms as to how T6SS affect EPS production and, thus, virulence. Identification of T6SS effectors in *E. amylovora* may further provide insight into the roles of T6SS in bacterial competition, EPS production, and virulence.

ACKNOWLEDGMENTS

This study was supported by the National Natural Science Foundation of China (number 31500107) and China Postdoctoral Science Foundation (project number 2015M570461).

LITERATURE CITED

- Aschtgen, M. S., Gavioli, M., Dessen, A., Llobès, R., and Cascales, E. 2010. The SciZ protein anchors the enteroaggregative *Escherichia coli* type VI secretion system to the cell wall. *Mol. Microbiol.* 75:886-899.
- Ayers, A. R., Ayers, S. B., and Goodman, R. N. 1979. Extracellular polysaccharide of *Erwinia amylovora*: A correlation with virulence. *Appl. Environ. Microbiol.* 38:659-666.
- Bellemann, P., Bereswill, S., Berger, S., and Geider, K. 1994. Visualization of capsule formation by *Erwinia amylovora* and assays to determine amylovoran synthesis. *Int. J. Biol. Macromol.* 16:290-296.
- Bingle, L. E. H., Bailey, C. M., and Pallen, M. J. 2008. Type VI secretion: A beginner's guide. *Curr. Opin. Microbiol.* 11:3-8.
- Blondel, C. J., Yang, H. J., Castro, B., Chiang, S., Toro, C. S., Zaldivar, M., Contreras, I., Andrews-Polymenis, H. L., and Santiviago, C. A. 2010. Contribution of the type VI secretion system encoded in SPI-19 to chicken colonization by *Salmonella enterica* serotypes Gallinarum and Enteritidis. *PLoS One* 5:e11724.
- Bönemann, G., Pietrosiuk, A., Diemand, A., Zentgraf, H., and Mogk, A. 2009. Remodelling of VipA/VipB tubules by ClpV-mediated threading is crucial for type VI protein secretion. *EMBO J.* 28:315-325.
- Boyer, F., Fichant, G., Berthod, J., Vandenbrouck, Y., and Attree, I. 2009. Dissecting the bacterial type VI secretion system by a genome wide *in silico* analysis: What can be learned from available microbial genomic resources? *BMC Genomics* 10:104.
- Bröms, J. E., Ishikawa, T., Wai, S. N., and Sjöstedt, A. 2013. A functional VipA-VipB interaction is required for the type VI secretion system activity of *Vibrio cholera* O1 strain A1552. *BMC Microbiol.* 13:96.
- Calvin, L., and Krissoff, B. 1998. Technical barriers to trade: A case study of phytosanitary barriers and U.S.-Japanese apple trade. *J. Agric. Resour. Econ.* 23:351-366.
- Christie, P. J., Amakuri, K., Krishnamoorthy, V., Jakubowski, S., and Cascales, E. 2005. Biogenesis, architecture, and function of bacterial type IV secretion systems. *Annu. Rev. Microbiol.* 59:451-485.
- Cornelis, G. R. 2006. The type III secretion injectisome. *Nat. Rev. Microbiol.* 4:811-825.
- De Maayer, P., Venter, S. N., Kamber, T., Duffy, B., Coutinho, T. A., and Smits, T. H. M. 2011. Comparative genomics of the type VI secretion systems of *Pantoea* and *Erwinia* species reveals the presence of putative effector islands that may be translocated by the VgrG and Hcp proteins. *BMC Genomics* 12:576.
- Denny, T. P. 1995. Involvement of bacterial polysaccharides in plant pathogenesis. *Annu. Rev. Phytopathol.* 33:173-197.
- Dong, T. G., Ho, B. T., Yoder-Himes, D. R., and Mekalanos, J. J. 2013. Identification of T6SS-dependent effector and immunity proteins by Tnseq in *Vibrio cholerae*. *Proc. Natl. Acad. Sci. USA* 110:2623-2628.
- Eastgate, J. A., Taylor, N., Coleman, M. J., Healy, B., Thompson, L., and Roberts, I. S. 1995. Cloning, expression, and characterization of the *lon*

- gene of *Erwinia amylovora*: Evidence for a heat shock response. *J. Bacteriol.* 177:932-937.
- Edmunds, A. C., Castiblanco, L. F., Sundin, G. W., and Waters, C. M. 2013. Cyclic Di-GMP modulates the disease progression of *Erwinia amylovora*. *J. Bacteriol.* 195:2155-2165.
- Filloux, A., Hachani, A., and Bleves, S. 2008. The bacterial type VI secretion machine: Yet another player for protein transport across membranes. *Microbiology* 154:1570-1583.
- Gross, M., Geier, G., Rudolph, K., and Geider, K. 1992. Levan and levansucrase synthesized by the fire blight pathogen *Erwinia amylovora*. *Physiol. Mol. Plant Pathol.* 40:371-381.
- Hachani, A., Lossi, N. S., and Filloux, A. 2013. A visual assay to monitor T6SS-mediated bacterial competition. *J. Vis. Exp.* 73:e50103.
- Hildebrand, M., Aldridge, P., and Geider, K. 2006. Characterization of *hns* genes from *Erwinia amylovora*. *Mol. Genet. Genomics* 275:310-319.
- Ho, B. T., Dong, T. G., and Mekalanos, J. J. 2014. A view to a kill: The bacterial type VI secretion system. *Cell Host Microbe* 15:9-21.
- Hoang, T. T., Karkhoff-Schweizer, R. R., Kutchma, A. J., and Schweizer, H. P. 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* 212:77-86.
- Hood, R. D., Singh, P., Hsu, F., Güvener, T., Carl, M. A., Trinidad, R. R., Silverman, J. M., Ohlson, B. B., Hicks, K. G., Plemel, R. L., Li, M., Schwarz, S., Wang, W. Y., Merz, A. J., Goodlett, D. R., and Mougous, J. D. 2010. A type VI secretion system of *Pseudomonas aeruginosa* targets a toxin to bacteria. *Cell Host Microbe* 7:25-37.
- Hsu, F., Schwarz, S., and Mougous, J. D. 2009. TagR promotes PpkA-catalysed type VI secretion activation in *Pseudomonas aeruginosa*. *Mol. Microbiol.* 72:1111-1125.
- Ishikawa, T., Sabharwal, D., Broms, J., Milton, D. L., Sjøstedt, A., Uhlin, B. E., and Wai, S. N. 2012. Pathoadaptive conditional regulation of the type VI secretion system in *Vibrio cholerae* O1 strains. *Infect. Immun.* 80:575-584.
- Jock, S., Donat, V., Lopez, M. M., Bazzi, C., and Geider, K. 2002. Following spread of fire blight in Western, Central and Southern Europe by molecular differentiation of *Erwinia amylovora* strains with PFGE analysis. *Environ. Microbiol.* 4:106-114.
- Kovach, M. E., Elzer, P. H., Hill, D. S., Robertson, G. T., Farris, M. A., Roop, R. M., 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.
- Leung, K. Y., Siame, B. A., Tenkink, B. J., Noort, R. J., and Mok, Y. K. 2011. Type VI secretion regulation: Crosstalk and intracellular communication. *Curr. Opin. Microbiol.* 14:9-15.
- Ma, L. S., Hachani, A., Lin, J., Filloux, A., and Lai, E. 2014. *Agrobacterium tumefaciens* deploys a superfamily of type IV secretion DNase effectors as weapons for interbacterial competition in planta. *Cell Host Microbe* 16: 94-104.
- Mann, R. A., Smits, T. H., Bühlmann, A., Blom, J., Goesmann, A., Frey, J. E., Plummer, K. M., Beer, S. V., Luck, J., Duffy, B., and Rodoni, B. 2013. Comparative genomics of 12 strains of *Erwinia amylovora* identifies a pan-genome with a large conserved core. *PLoS One* 8:e55644.
- Matthysse, A. G., Yarnall, H. A., and Young, N. 1996. Requirement for genes with homology to ABC transport systems for attachment and virulence of *Agrobacterium tumefaciens*. *J. Bacteriol.* 178:5302-5308.
- Mattinen, L., Nissinen, R., Riipi, T., Kalkkinen, N., and Pirhonen, M. 2007. Host-extract induced changes in the secretome of the plant pathogenic bacterium *Pectobacterium atrosepticum*. *Proteomics* 7:3527-3537.
- McNally, R. R., Toth, I. K., Cock, P. J., Pritchard, L., Hedley, P. E., Morris, J. A., Zhao, Y., and Sundin, G. W. 2012. Genetic characterization of the *HrpL* regulation of the fire blight pathogen *Erwinia amylovora* reveals novel virulence factors. *Mol. Plant Pathol.* 13:160-173.
- Mougous, J. D., Cuff, M. E., Raunser, S., Shen, A., Zhou, M., Gifford, C. A., Goodman, A. L., Joachimiak, G., Ordoñez, C. L., Lory, S., Walz, T., Joachimiak, A., and Mekalanos, J. J. 2006. A virulence locus of *Pseudomonas aeruginosa* encodes a protein secretion apparatus. *Science* 312:1526-1530.
- Myung, I.-S., Lee, J.-Y., Yun, M.-J., Lee, Y.-H., Lee, Y.-K., Park, D.-H. and Oh, C.-S. 2016. Fire blight of apple, caused by *Erwinia amylovora*, a new disease in Korea. *Plant Dis.* 100:1774.
- Ordax, M., Marco-Noales, E., López, M. M., and Biosca, E. G. 2010. Exopolysaccharides favor the survival of *Erwinia amylovora* under copper stress through different strategies. *Res. Microbiol.* 161:549-555.
- Pukatzki, S., Ma, A. T., Revel, A. T., Sturtevant, D., and Mekalanos, J. J. 2007. Type VI secretion system translocates a phage tail spike-like protein into target cells where it cross-links actin. *Proc. Natl. Acad. Sci. USA* 104:15508-15513.
- Pukatzki, S., Ma, A. T., Sturtevant, D., Krastins, B., Sarracino, D., Nelson, W. C., Heidelberg, J. F., and Mekalanos, J. J. 2006. Identification of a conserved bacterial protein secretion system in *Vibrio cholera* using the *Dictyostelium* host model system. *Proc. Natl. Acad. Sci. USA* 103:1528-1533.
- Pukatzki, S., McAuley, S. B., and Miyata, S. T. 2009. The type VI secretion system: Translocation of effectors and effector-domains. *Curr. Opin. Microbiol.* 12:11-17.
- Records, A. R. 2011. The type VI secretion system: A multipurpose delivery system with a phage-like machinery. *Mol. Plant-Microbe Interact.* 24:751-757.
- Records, A. R., and Gross, D. C. 2010. Sensor kinases RetS and LadS regulate *Pseudomonas syringae* type VI secretion and virulence factors. *J. Bacteriol.* 192:3584-3596.
- Sarris, P. F., Trantas, E. A., Skandalis, N., Tampakaki, A. P., Kapanidou, M., Kokkinidis, M., and Panopoulos, N. J. 2012. Phyto-bacterial type VI secretion system—Gene distribution, phylogeny, structure and biological functions. Pages 53-84 in: *Plant Pathology*. C. J. R. Cumagun, ed. InTech.
- Schwarz, S., Hood, R. D., and Mougous, J. D. 2010. What is type VI secretion doing in all those bugs? *Trends Microbiol.* 18:531-537.
- Segal, G., Feldman, M., and Zusman, T. 2005. The Icm/Dot type-IV secretion systems of *Legionella pneumophila* and *Coxiella burnetii*. *FEMS Microbiol. Rev.* 29:65-81.
- Shneider, M. M., Buth, S. A., Ho, B. T., Basler, M., Mekalanos, J. J., and Leiman, P. G. 2013. PAAR-repeat proteins sharpen and diversify the type VI secretion system spike. *Nature* 500:350-353.
- Shyntum, D. Y., Theron, J., Venter, S. N., Moleleki, L. N., Toth, I. K., and Coutinho, T. A. 2015. *Pantoea ananatis* utilizes a type VI secretion system for pathogenesis and bacterial competition. *Mol. Plant-Microbe Interact.* 28:420-431.
- Silverman, J. M., Austin, L. S., Hsu, F., Hicks, K. G., Hood, R. D., and Mougous, J. D. 2011. Separate inputs modulate phosphorylation-dependent and -independent type VI secretion activation. *Mol. Microbiol.* 82:1277-1290.
- Simon, R., Priefer, U., and Pühler, A. 1983. A broad host range mobilization system for *in vivo* genetic engineering: Transposon mutagenesis in gram negative bacteria. *Nat. Biotechnol.* 1:784-791.
- Sjulin, T. M., and Beer, S. V. 1978. Mechanism of wilt induction by amylo-
voran in Cotoneaster shoots and its relation to wilting of shoots infected by *Erwinia amylovora*. *Phytopathology* 68:89-94.
- Smits, T. H. M., Rezzonico, F., Kamber, T., Blom, J., Goesmann, A., Frey, J. E., and Duffy, B. 2010. Complete genome sequence of the fire blight pathogen *Erwinia amylovora* CFBP 1430 and comparison to other *Erwinia* spp. *Mol. Plant-Microbe Interact.* 23:384-393.
- Steinberger, E. M. 1988. Creation and complementation of pathogenicity mutants of *Erwinia amylovora*. *Mol. Plant-Microbe Interact.* 1:135-144.
- Suarez, G., Sierra, J. C., Sha, J., Wang, S., Erova, T. E., Fadl, A. A., Foltz, S. M., Horneman, A. J., and Chopra, A. K. 2008. Molecular characterization of a functional type VI secretion system from a clinical isolate of *Aeromonas hydrophila*. *Microb. Pathog.* 44:344-361.
- Tian, Y., Zhao, Y., Wu, X., Liu, F., Hu, B., and Walcott, R. R. 2015. The type VI protein secretion system contributes to biofilm formation and seed-to-seedling transmission of *Acidovorax citrulli* on melon. *Mol. Plant Pathol.* 16:38-47.
- van der Zwet, T. 2006. Present worldwide distribution of fire blight and closely related disease. *Acta Hort.* 704:35-36.
- Vanneste, J. L., ed. 2000. *Fire Blight: The Disease and Its Causative Agent, Erwinia amylovora*. CABI Publishing, New York.
- Vrancken, K., Holtappels, M., Schoofs, H., Deckers, T., and Valcke, R. 2013. Pathogenicity and infection strategies of the fire blight pathogen *Erwinia amylovora* in Rosaceae: State of the art. *Microbiology* 159:823-832.
- Wang, D., Korban, S. S., Pusey, P. L., and Zhao, Y. 2012. *AmyR* is a novel negative regulator of amylo-
vran production in *Erwinia amylovora*. *PLoS One* 7:e45038.
- Weber, B., Hasic, M., Chen, C., Wai, S. N., and Milton, D. L. 2009. Type VI secretion modulates quorum sensing and stress response in *Vibrio anguillarum*. *Environ. Microbiol.* 11:3018-3028.
- Wu, H. Y., Chung, P. C., Shih, H. W., Wen, S. R., and Lai, E. M. 2008. Secretome analysis uncovers an Hcp-family protein secreted via a type VI secretion system in *Agrobacterium tumefaciens*. *J. Bacteriol.* 190:2841-2850.
- Zeng, Q., McNally, R. R., and Sundin, G. W. 2013. Global small RNA chaperone Hfq and regulatory small RNAs are important virulence regulators in *Erwinia amylovora*. *J. Bacteriol.* 195:1706-1717.
- Zhang, H. B., Wang, L. H., and Zhang, L. H. 2002. Genetic control of quorum-sensing signal turnover in *Agrobacterium tumefaciens*. *Proc. Natl. Acad. Sci. USA* 99:4638-4643.
- Zhao, Y., Wang, D., Nakka, S., Sundin, G. W., and Korban, S. S. 2009. Systems-level analysis of two-component signal transduction systems in *Erwinia amylovora*: Role in virulence, regulation of amylo-
vran bio-synthesis and swarming motility. *BMC Genomics* 10:245.
- Zheng, J., Ho, B., and Mekalanos, J. J. 2011. Genetic analysis of anti-amoe-
bae and anti-bacterial activities of the type VI secretion system in *Vibrio cholerae*. *PLoS One* 6:e23876.
- Zheng, J., and Leung, K. Y. 2007. Dissection of a type VI secretion system in *Edwardsiella tarda*. *Mol. Microbiol.* 66:1192-1206.
- 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.