Pantoea ananatis utilizes a type VI secretion system for pathogenesis and bacterial competition

Divine Y. Shyntum,[1,2] Jacques Theron,[1] Stephanus N. Venter,[1,2] Lucy N. Moleleki,[1,2] Ian K. Toth,[2,3] and Teresa A. Coutinho[1,2]

[1]Department of Microbiology and Plant Pathology, Faculty of Natural and Agricultural Sciences, University of Pretoria, Pretoria 0002, South Africa.

[2]Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria 0002, South Africa.

[3]James Hutton Research Institute, Invergowrie, Dundee DD2 5DA, Scotland, United Kingdom.

Corresponding Author: Prof Teresa A. Coutinho

E-mail: Teresa.Coutinho@up.ac.za

Tel: +27 12 420-3934

Fax: +27 12 420-3960

ABSTRACT

Type VI secretion systems (T6SSs) are a class of macromolecular machines that are recognized as an important virulence mechanism in several Gram-negative bacteria. The genome of *Pantoea ananatis* LMG 2665^T, a pathogen of pineapple fruit and onion plants, carries two gene clusters whose predicted products have homology with T6SS-associated gene products from other bacteria. Nothing is known regarding the role of these T6SS-1 and T6SS-3 gene clusters in the biology of *P. ananatis*. Here, we present evidence that T6SS-1 plays an important role in the pathogenicity of *P. ananatis* LMG 2665^T in onion plants, while a strain lacking T6SS-3 remains as pathogenic as the wild-type strain. We also investigated the role of the T6SS-1 system in bacterial competition, the results of which indicated that several bacteria compete less efficiently against wild-type LMG 2665^T than a strain lacking T6SS-1. Additionally, we demonstrated that these phenotypes of strain LMG 2665^T were reliant on the core T6SS products TssA and TssD (Hcp), thus indicating that the T6SS-1 gene cluster encodes a functioning T6SS. Collectively, our data provides the first evidence demonstrating that the T6SS-1 system is a virulence determinant of *P. ananatis* LMG 2665^T and plays a role in bacterial competition.

INTRODUCTION

Secretion of proteins such as extracellular proteases and toxins can provide selective advantages to bacteria in various environmental niches, and many of the proteins secreted by pathogenic bacteria are important colonization and virulence factors. To date, six types of protein secretion systems (type I through type VI secretion system [T1SS through T6SS]) have been described in Gram-negative bacteria (Economou et al. 2006; Holland 2010). These

secretion systems are distinguished by the conserved structural components that define them, as well as the characteristics of their substrates and the molecular mechanisms underlying the export process. The most recently described T6SS has emerged as having a role in bacterial pathogenesis and host interactions (Coulthurst 2013; Kapitein and Mogk 2013). Data from structural studies, functional assays and protein localization studies suggest that the T6SS consists of a membrane-associated assembly platform and a cell surface-exposed needle structure that transports effector molecules into bacteria or eukaryotic cells (Filloux et al. 2008; Silverman et al. 2012).

Whole-genome analyses have predicted T6SS gene clusters to be widely distributed in Gram-negative bacterial species (Bingle et al. 2008; Boyer et al. 2009). Although the T6SS gene clusters differ between bacterial species in terms of gene order and composition, they are comprised of at least 13 core genes (*tss*, nomenclature proposed by Shalom et al. [2007]) and a variable number of non-conserved accessory elements that encode the T6SS "injectisome" (Bingle et al. 2008; Cascales 2008). A number of the T6SS core proteins are evolutionary and structurally related to bacteriophage proteins (Kanamura et al. 2009; Leiman et al. 2009). Examples are the baseplate gp25-like protein TssE, the tail sheath-like proteins TssB and TssC, the tail subunit-like hemolysin co-regulated protein (Hcp; TssD) that polymerizes into the T6SS needle structure, and the valine-glycine repeat protein G (VgrG; TssI) that forms the spike of the TssD nanotube (Ballister et al. 2008; Cascales and Cambillau 2012; Lossi et al. 2011; Lossi et al. 2013; Pukatzki et al. 2007). Contraction and extension of the TssB-TssC tubular sheath of the T6SS of *Vibrio cholerae* have been visualized *in vivo*, suggesting that the T6SS sheath is a dynamic contractile structure that projects the T6SS spike into the target cell analogous to the bacteriophage infection process (Basler et al. 2012). Disassembly of the contracted sheath requires the ClpV (TssH) AAA+ ATPase, which binds specifically to the

contracted TssB-TssC sheath for its disassembly and cycling (Böneman et al. 2009; Kapitein et al. 2013). Another group of T6SS building blocks (TssM-L) appears to be related to proteins of the T4SS (Durand et al. 2012; Felisberto-Rodrigues et al. 2011) and may be involved in the recruitment of TssD (Hcp) to the T6SS inner membrane assembly platform (Ma et al. 2012).

The T6SSs have been implicated in a variety of functions ranging from biofilm formation to host-cell invasion, cytotoxicity and survival in macrophages (Aschtgen et al. 2008; Cascales 2008; Schwarz et al. 2010a). However, most studies of the T6SS have focused on its role in pathogenesis and host interactions. The T6SS has been implicated as a virulence factor in several human or animal pathogens, including *Vibrio cholerae* (Pukatzki et al. 2006), *Pseudomonas aeruginosa* (Mougous et al. 2006), *Burkholderia mallei* (Schell et al. 2007), *Aeromonas hydrophila* (Suarez et al. 2008), *Edwardsiella tarda* (Zheng and Leung 2007), *Salmonella enterica* serovar Gallinarum (Blondel et al. 2010), and avian pathogenic *Escherichia coli* (de Pace et al. 2010). It has subsequently been revealed that some T6SSs are used to target other bacteria, efficiently killing or inhibiting the growth of competitors, as reported for T6SSs of *Serratia marcescens* (Murdoch et al. 2011), *P. aeruginosa* (Hood et al. 2010), *Burkholderia thailandensis* (Schwarz et al. 2010b), and *V. cholerae* (MacIntyre et al. 2010). In contrast to animal and human pathogens, the role of T6SSs in plant bacterial pathogens is still largely unknown (Records, 2011). Nevertheless, T6SS functionality has been demonstrated for a few plant-associated bacteria, including *Agrobacterium tumefaciens* (Lin et al. 2013; Wu et al. 2008), *Pectobacterium atrosepticum* (Liu et al. 2008) and *Pseudomonas syringae* (Haapalainen et al. 2012), and it was recently reported that the T6SS of *Pseudomonas fluorescens* plays an important role in bacterial competition (Decoin et al. 2014).

Pantoea ananatis is a Gram-negative bacterial pathogen of plants. It causes disease in a wide variety of economically important plants such as *Eucalyptus* spp., Sudangrass, cotton, rice, corn, onion, melon, cantaloupe fruit, and pineapple (Coutinho and Venter 2009). Diseases caused by *P. ananatis* in onion, for example, result in reductions in crop yield, thus leading to substantial economic losses (Gitaitis and Gay 1997; Goszczynska et al. 2007; Walcott et al. 2002). To date, there are a limited number of reports that focus on the mechanisms by which *P. ananatis* causes disease (Morohoshi et al. 2007; Morohoshi et al. 2011a; Morohoshi et al. 2011b; Sessitsch et al. 2004). Consequently, the pathogenesis of *P. ananatis* is still poorly understood and the potential virulence determinants and mechanisms employed by *P. ananatis* have yet to be defined. Comparative genomic analysis of *Pantoea* species have demonstrated the presence of up to three gene clusters, designated T6SS-1 through T6SS-3, encoding components of the T6SS (De Maayer et al. 2011). Subsequent comparative genomics of sequenced *P. ananatis* strains indicated that the T6SS-1 and T6SS-3 gene clusters are present in all strains analyzed, whereas the T6SS-2 gene cluster is present in some but not all of these strains (Shyntum et al. 2014). It is currently not known whether these gene clusters are functionally redundant or are required for a specific activity.

In this study, we made use of a targeted mutagenesis strategy to evaluate the T6SS-1 and T6SS-3 gene clusters, present in the genome of *P. ananatis* LMG 2665^{T}, for a role in pathogenesis and competitiveness. The results indicate that T6SS-1 is an important virulence determinant of *P. ananatis* LMG 2665^{T}, and plays a role in intra- and interspecies bacterial competition.

RESULTS

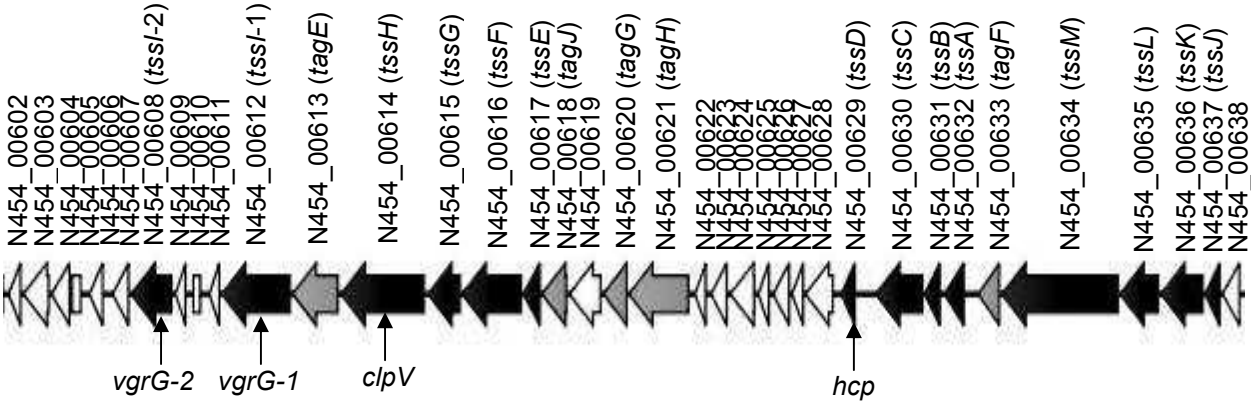
Construction of T6SS gene cluster deletions in *P. ananatis* LMG 2665^T.

Previous sequence analyses demonstrated the presence of two gene clusters in the genome of *P. ananatis* LMG 2665^T that contain genes homologous to those present in T6SSs (Shyntum et al. 2014). The 40.6-kb T6SS-1 gene cluster contains genes that are predicted to encode the 13 core T6SS proteins (TssA-M), five proteins associated with T6SSs in other bacteria (Tag) and 18 proteins that are present in very few or no other systems. In contrast, the 8.4-kb T6SS-3 gene cluster encodes two proteins (TssM and TssL) that are conserved in T6SSs and four accessory proteins (Fig. 1). No genetic analysis of these loci has been performed previously, and the function of the proteins encoded in these loci has also not yet been explored. Thus, we began by deleting the individual putative T6SS loci of *P. ananatis* LMG 2665^T. In this study, we used the lambda Red-recombineering technique (Datsenko and Wanner 2000) to delete the gene clusters and replace them with a kanamycin resistance cassette, yielding strains 2665^{T}ΔT6SS-1 and 2665^{T}ΔT6SS-3, respectively. The growth curves of the wild-type LMG 2665^T strain and the mutant strains in LB broth and *in planta* were similar (Fig. 2), indicating that deletion of the respective gene clusters did not alter growth kinetics.

The T6SS-1 of *P. ananatis* LMG 2665^T is required for pathogenesis in onion plants.

To determine whether the T6SS gene clusters play a role in the biology of *P. ananatis*, we assessed the ability of mutants lacking the T6SS-1 or T6SS-3 gene clusters to cause disease by conducting pathogenicity tests on susceptible onion plants. The wild-type strain LMG 2665^T and its mutants were inoculated into onion leaves and the development of disease symptoms was monitored. Although the 2665^{T}ΔT6SS-1 mutant strain did not induce any disease symptoms, onion leaves infected with the wild-type LMG 2665^T strain or the

T6SS-1 gene cluster



T6SS-3 gene cluster

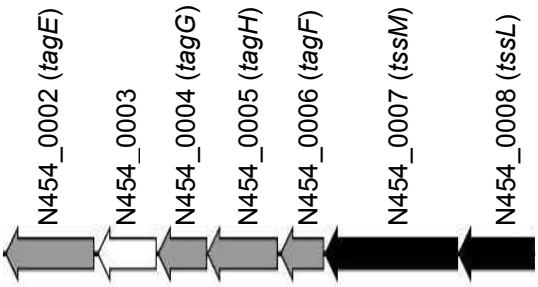


Fig. 1. The putative T6SS gene clusters of P. ananatis LMG 2665T. The genes (indicated with locus names) with homology to conserved core T6SS components are designated as tss (type VI secretion) and indicated in black, whereas genes associated with T6SSs of several bacteria are designated as tag (type VI secretion-associated gene) and indicated in grey. The nomenclature is based on that proposed by Shalom et al. (2007).

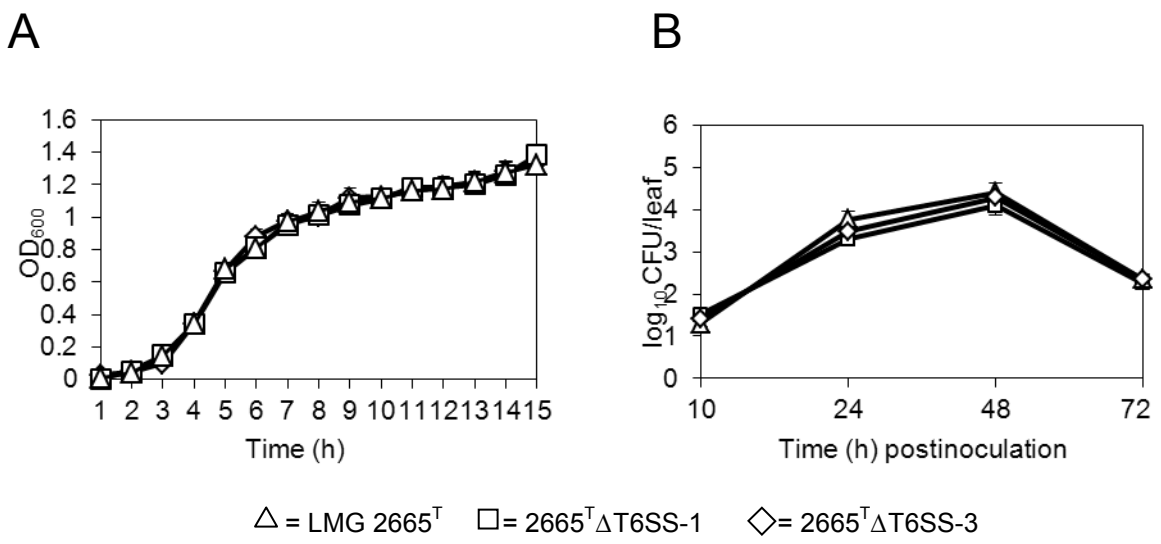


Fig. 2. Growth of P. ananatis wild-type LMG 2665T and mutant strains lacking the T6SS-1 or T6SS-3 gene clusters. (A) The strains were cultured in LB broth at 32°C and growth was monitored for 15 h at OD600. (B) Prior to in planta growth determination, the P. ananatis wild-type and mutant strains were transformed with plasmid pMP7605 to confer gentamycin resistance. Onion leaves were inoculated with 103 bacteria of the derivative wild-type and mutant strains, and the plants were incubated in a greenhouse at 28 to 30°C. At the indicated times postinoculation, leaves were cut, homogenized and the CFU determined by plating on LB agar medium with antibiotic. In both A and B, the results are presented as the mean of three independent experiments and the error bars represent the standard error of the mean.

2665^{T}ΔT6SS-3 mutant strain developed symptoms typical of disease caused by *P. ananatis* in onion plants. Initially, the onion leaves infected with strain LMG 2665^T or 2665^{T}ΔT6SS-3 developed water-soaked spots on the sites of inoculation, which was followed by complete collapse of the infected leaves, necrosis, wilting and then death (Fig. 3A). At 3 days postinoculation, the number of wilted (dead) leaves per plant for each strain was recorded and the average percentage of dead leaves calculated. On average, the mutant 2665^{T}ΔT6SS-1 strain was significantly ($P < 0.05$) reduced in virulence compared to the wild-type LMG 2665^T strain, whereas the mutant 2665^{T}ΔT6SS-3 strain retained virulence levels similar to those of the wild-type strain (Fig. 3B). These data demonstrate that the pathogenicity of *P. ananatis* LMG 2665^T is dependent on the presence of the T6SS-1 but not the T6SS-3 gene cluster.

The T6SS-1 of *P. ananatis* LMG 2665^T is used to compete against different Gram-negative bacteria.

An increasing number of T6SSs have been linked to interbacterial killing of Gram-negative bacteria through the delivery of different toxins that target the peptidoglycan of susceptible bacterial species (Carruthers et al. 2013; English et al. 2012; Hood et al. 2010; Russell et al. 2011). To determine whether *P. ananatis* LMG 2665^T displays antibacterial activity and also to explore the scope of the potential antibacterial activity, an *in vitro* competition assay was performed. The wild-type LMG 2665^T strain was initially tested against a panel of 30 Gram-negative bacteria, including *E. coli*, which has previously been shown to be susceptible to T6SS killing (MacIntyre et al. 2010; Weber et al. 2013; Zheng et al. 2011). Gram-positive bacteria (*Bacillus* spp. and *B. cereus*) were included in the assay as controls (Table S2). The wild-type LMG 2665^T strain was virulent towards various Gram-negative bacteria, but did not display antimicrobial activity towards any of the Gram-positive bacteria tested (data not

A

B

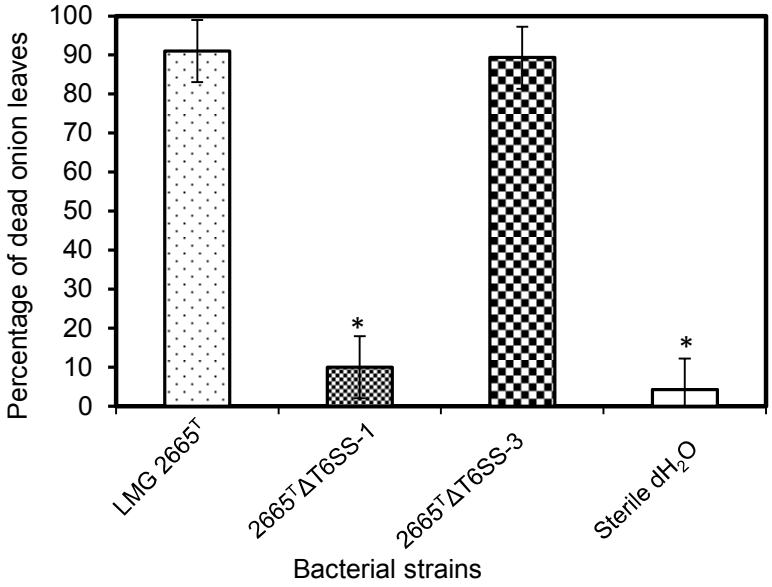


Fig. 3. Pathogenicity of P. ananatis wild-type LMG 2665T and mutant strains lacking the T6SS-1 or T6SS-3 gene clusters. The leaves of six-week-old onion plants were inoculated with 103 bacteria at one site per leaf and the plants were incubated in a greenhouse for 3 days. In these assays, plants inoculated with sterile distilled water (dH2O) were included as a negative control. Three individual experiments, each containing at least 20 plants per treatment, were performed. (A) Disease symptoms of onion plants inoculated with the P. ananatis strains. The pictures were taken at 3 days postinoculation and indicate representative results. (B) At 3 days postinoculation, the number of inoculated wilted (dead) leaves per onion plant was recorded and the percentage of dead leaves was calculated. Data represent the mean percentage of dead leaves from the three biological repeats and the error bars represent the standard error of the mean. Statistically significant differences between P.ananatis LMG 2665T and the respective mutant strains was determined by an unpaired, twotailed Student's t-test, and are indicated by asterisks.

shown). The antibacterial activity of the LMG 2665^T strain was limited to *E. coli* DH5α, *P. carotovorum* subsp. *carotovorum* LMG 2404^T, *Salmonella enterica* serovar Typhimurium, *Pantoea stewartii* subsp. *indologenes*, and two strains of *P. ananatis* (LMG 2669 and LMG 2664). These Gram-negative bacteria were used in all subsequent competition experiments.

To test the contribution of the T6SS gene clusters to the antibacterial properties of *P. ananatis* LMG 2665^T, we examined whether the mutants lacking the T6SS-1 or T6SS-3 gene clusters could reduce the numbers of the above bacteria when grown in competition on agar plates. When each competitor strain was cocultured with wild-type LMG 2665^T or the 2665^{T}ΔT6SS-3 mutant strain, there was a significant ($P < 0.05$) drop in the number of viable cells recovered (10- to 100-fold) compared with results for the no-treatment controls. However, when the coculture was with 2665^{T}ΔT6SS-1, the survival of competitor bacteria was increased up to 100-fold compared to that of wild-type LMG 2665^T or 2665^{T}ΔT6SS-3 (Fig. 4). Overall, the results suggest that the T6SS-1 gene cluster provides *P. ananatis* LMG 2665^T with a competitive advantage towards selected Gram-negative bacteria.

Construction of *tssA* and *tssD* (*hcp*) mutants of *P. ananatis* LMG 2665^T.

To determine whether *P. ananatis* LMG 2665^T produces a functioning T6SS, we selected the *tssA* and *tssD* (*hcp*) genes present in the T6SS-1 gene cluster for mutational analyses. TssD (Hcp), a "hallmark" of T6SSs (Bingle et al. 2008), forms hexameric rings that polymerize into tubules. It is believed that these nanotubes are extruded following contraction of the surrounding TssB-TssC sheath, thereby facilitating transport of T6SS-dependent effector proteins across membranes of target cells (Ballister et al. 2008; Basler et al. 2012; Jobichen et al. 2010). TssA is predicted to be a cytoplasmic protein and contains an ImpA-like domain of unknown function (Cascales and Cambillou 2012). It has been speculated that TssA plays a

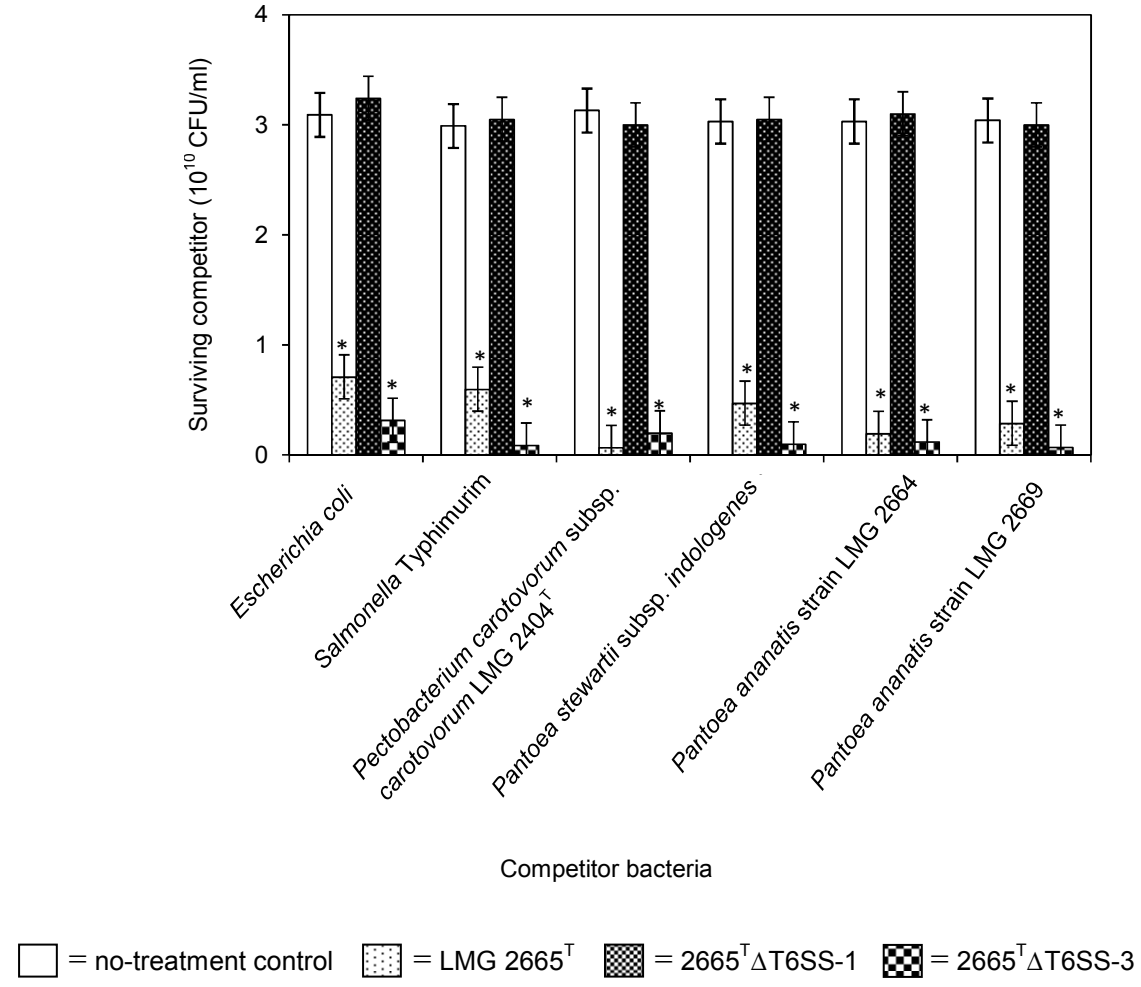


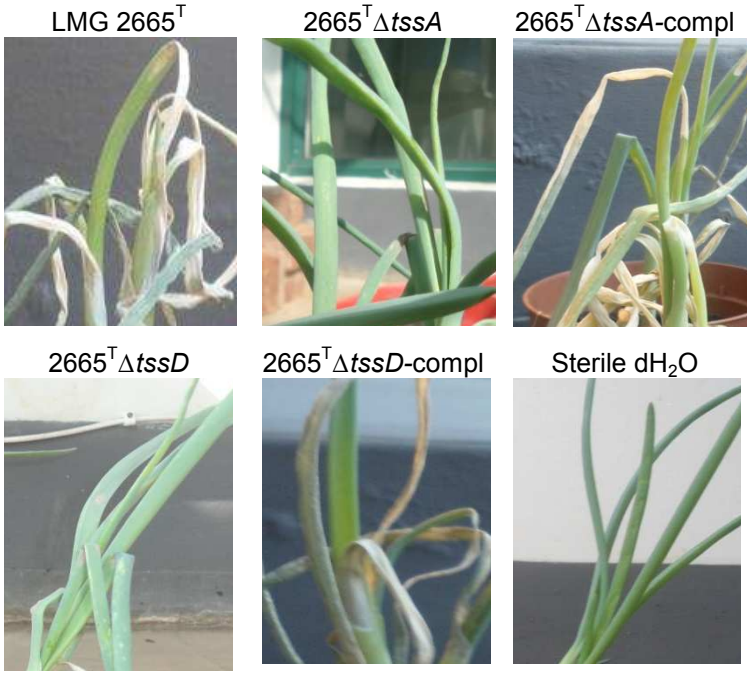
Fig. 4. The T6SS-1 of P. ananatis wild-type LMG 2665T is used for antibacterial activity. The P. ananatis wild-type, 2665TKT6SS-1 or 2665TKT6SS-3 mutant strains were mixed with a gentamycin-resistant bacterium at a ratio of 1:1, spotted onto LB agar and after overnight incubation, spots were recovered and survivor competitor bacterial cells were assessed by spreading dilutions on LB agar with antibiotic and CFU determination. Data represent the mean CFU/ml from three independent experiments and error bars represent the standard error of the mean. The CFU/ml of competitor bacteria was significantly reduced in competition assays with strains LMG 2665T and 2665TKT6SS-3 ($P < 0.05$; unpaired, two-tailed Student's t-test), but not with strain 2665TKT6SS-1, when compared to the no-treatment controls. Statistically significant differences are denoted with asterisks.

regulatory role or is associated with proteins destined for secretion (Shrivastava and Monde 2008). The *P. ananatis* LMG 2665^T mutant strains were constructed by replacement of the selected genes with a kanamycin resistant cassette, yielding strains 2665^{T}Δ*tssA* and 2665^{T}Δ*tssD*, respectively. As expected from deletion of the entire T6SS-1 cluster (see above), deletion of the *tssA* or *tssD* genes did not have any detectable impact on growth *in vitro* and *in planta* (data not shown).

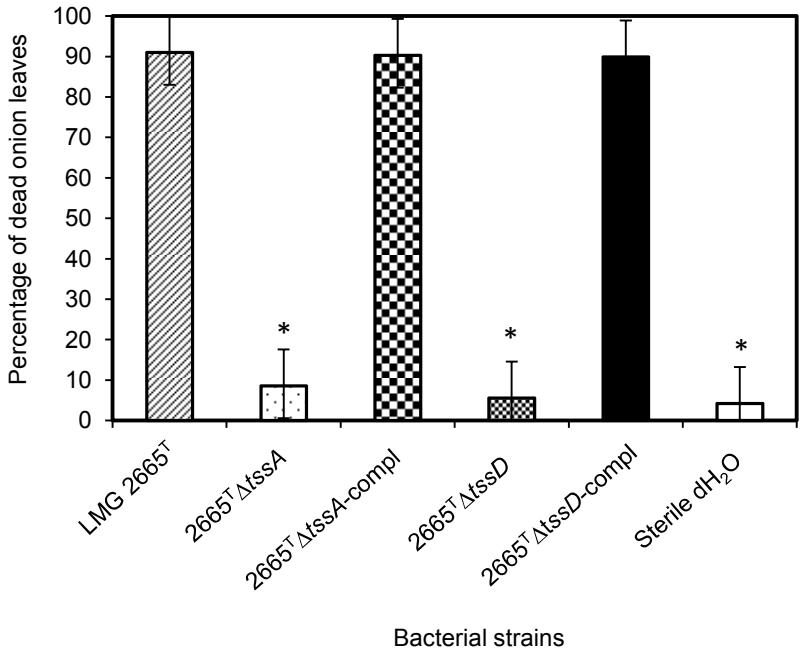
The T6SS-1 of *P. ananatis* LMG 2665^T encodes a functioning T6SS.

To determine the effects of the *tssA* and *tssD* gene deletions on T6SS-1 activity, we examined each mutant strain for their contributions to pathogenicity in onion plants and antibacterial activity. The pathogenicity of the 2665^{T}Δ*tssA* and 2665^{T}Δ*tssD* mutant strains was compared to the wild-type LMG 2665^T strain by conducting pathogenicity tests on onion leaves, as described above. The results indicated that in contrast to the wild-type strain, neither of the mutant strains induced disease (Figs. 5A and 5B). We also repeated the bacterial competition assay with the Gram-negative bacterial strains previously shown to be susceptible to T6SS-1-dependent antibacterial activity. After competition with the wild-type strain LMG 2665^T a 10- to 100-fold reduction ($P < 0.05$) in bacteria was observed after coculture, while surviving bacterial populations from competitions with 2665^{T}Δ*tssA* and 2665^{T}Δ*tssD* were equivalent to no-treatment controls (Fig. 5C). To directly link the phenotypes observed for the 2665^{T}Δ*tssA* and 2665^{T}Δ*tssD* mutant strains to T6SS-1 functionality, the respective mutant strains were transformed with a plasmid harboring a wild-type copy of the *tssA* or *tssD* genes. As shown in Fig. 5, introduction of the *tssA* and *tssD* genes *in trans* restored the activity of the 2665^{T}Δ*tssA* and 2665^{T}Δ*tssD* strains to cause disease in onion plants and to compete with bacteria, thus demonstrating that the mutations are not polar and that these genes are required to produce a functional T6SS apparatus in *P. ananatis* LMG 2665^T.

A


LMG 2665T
2665TΔtssA
2665TΔtssA-compl
2665TΔtssD
2665TΔtssD-compl
Sterile dH2O

B


Percentage of dead onion leaves
100
90
80
70
60
50
40
30
20
10
0
*
*
*
LMG 2665T
2665TΔtssA
2665TΔtssA-compl
2665TΔtssD
2665TΔtssD-compl
Sterile dH2O
Bacterial strains

C

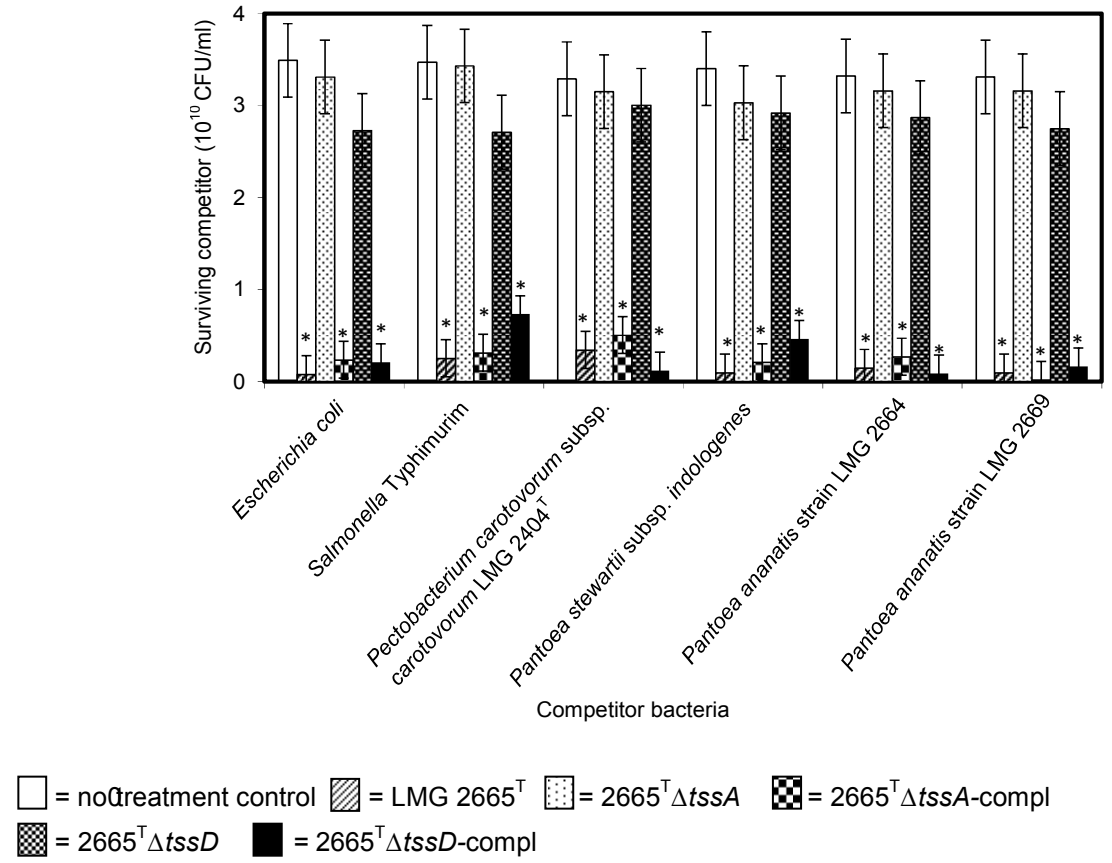


Fig. 5. Phenotypic analysis of P. ananatis LMG 2665T mutant strains lacking the T6SS-1 gene cluster genes tssA and tssD. (A) Disease symptoms of onion plants inoculated with the wild-type LMG 2665T, mutant or complemented mutant strains. Representative pictures, taken at 3 days postinoculation, are shown. (B) Virulence of the wild-type and complemented mutant strains was not significantly different, although these strains differed significantly from the mutant strains. The bars represent the mean percentage of dead of onion leaves from three individual experiments, each containing at least 20 plants per treatment, and error bars represent the standard error of the mean. Statistically significant differences between the P.ananatis strains were determined by an unpaired, two-tailed Student's t-test, and are indicated by asterisks. (C) In bacterial competition assays, the survival of competitor bacteria was determined by measuring the corresponding CFU after exposure to either the wild-type, mutant or complemented mutant strains of P. ananatis LMG 2665T. Data represent CFU/ml from three independent experiments. The bars represent mean values and error bars denote the standard error of the mean. Statistically significant differences between the respective P.ananatis LMG 2665T strains and the no-treatment controls were determined by an unpaired, two-tailed Student's t-test, and are indicated by asterisks.

DISCUSSION

Protein secretion systems are often critical to the virulence and host-interaction processes of Gram-negative bacterial pathogens (Gerlach and Hensel 2007). Amongst the different secretion systems, the T2SS secretes proteins from the bacteria to the exterior to degrade host cell components (Cianciotto 2005; Sandkvist 2001), whereas the T3SS and T4SS transfer effectors directly from the bacteria into host cells and, consequently, manipulate the host response for their own benefit (Backert and Meyer 2006; Cornelis 2006; Hueck 1998). Despite its ability to cause disease in a wide variety of economically important crops, *P. ananatis* lacks genes homologous to the above-mentioned secretion systems (De Maayer et al. 2011). Thus, the strategy and mechanism(s) that contribute to infection and disease development are poorly understood in this plant pathogen. Notably, T6SS-associated genes have been identified in *Pantoea* spp. (De Maayer et al. 2011) and *P. ananatis* (Shyntum et al. 2014) specifically. In the case of *P. ananatis* LMG 2665^{T}, the T6SS-associated genes are located in two clusters, named as T6SS-1 and T6SS-3, respectively. Considering that T6SSs have been implicated in promoting virulence and cytotoxicity in eukaryotic and bacterial hosts (Kapitein and Mogk 2013; Schwartz et al. 2010a), this has generated several questions as to whether the respective gene clusters in *P. ananatis* encode a functional T6SS and whether these T6SSs may play similar roles in the biology of *P. ananatis* LMG 2665^{T}.

Pathogenicity assays in onion plants, performed with *P. ananatis* LMG 2665^{T} mutant strains lacking the T6SS-1 or T6SS-3 gene clusters, showed that mutant strain 2665^{T}ΔT6SS-3 was as pathogenic as the wild-type LMG 2665^{T} strain, while mutant strain 2665^{T}ΔT6SS-1 was not pathogenic. These results indicate that the T6SS-1 gene cluster likely encodes a functional T6SS that has an essential role in pathogenicity. Although the truncated T6SS-3 gene cluster,

which lacks 11 of the core T6SS genes, appears not to play a role in either pathogenicity or antibacterial competition, and therefore may not encode a functional T6SS, it is intriguing to understand why this cluster is maintained. Its 100% prevalence amongst *P. ananatis* strains (Shyntum et al. 2014) suggests that this seemingly stable gene cluster may be advantageous to the bacteria for an as yet unknown function.

It is interesting to note that the T6SS-3 gene cluster is predicted to encode homologues of Fha (TagH), PpkA (TagE) and PppA (TagG), which have been implicated in the regulation of T6SS activity by a posttranslational protein phosphorylation mechanism. In *P. aeruginosa*, Fha is phosphorylated by the serine-threonine kinase PpkA and dephosphorylated by the phosphatase PppA, and the phosphorylation of Fha regulates the activity of the T6SS (Mougous et al. 2007). Considering that these posttranslational regulatory components are also encoded by the T6SS-1 gene cluster of *P. ananatis* LMG 2665^{T}, the implications of this potential redundancy are intriguing. Not only is it tempting to speculate that activation of the T6SS-1 of strain LMG2665^{T} depends on a similar posttranslational mechanism, but also that the products of the T6SS-3 gene cluster may contribute to differentially regulating T6SS-1 activity under different culture conditions. This may explain why the *P. ananatis* strains retain the truncated T6SS-3 gene cluster. Further work, however, will be required to clarify this hypothesis and to identify environmental signals that may be responsible for triggering the expression of the respective gene clusters.

Very little information is available about potential effector proteins that are secreted in a T6SS-dependent manner into eukaryotic cells (Miyata et al. 2011; Zheng and Leung 2007). In some cases, VgrG proteins can exert effector functions on eukaryotic cells. For these so-called evolved VgrG proteins, this function is typically associated with the presence of an

additional C-terminal effector domain. Activities of these evolved VgrGs include cross-linking or ADP-ribosylation of actin in eukaryotic cells, thereby promoting host cell toxicity (Ma and Mekalanos 2010; Pukatzki et al. 2007; Suarez et al. 2010). As shown in Fig. 1, the T6SS-1 gene cluster of *P. ananatis* LMG 2665^T contains two VgrG (TssI) homologues. The putative VgrG proteins lack C-terminal effector domains and are thus likely not essential for pathogenesis in onion plants. Indeed, in the case of *P. fluorescens* pv. *tomato*, which also contains two VgrG homologues lacking recognizable evolved C-terminal domains, it was reported that the VgrG-1 and VgrG-2 deletion mutant strains had no effect on disease development in tomato or in *Nicotiana benthamiana* (Sarris et al. 2012). A recent study, however, suggested that adaptor proteins may be widely utilized to facilitate the recruitment of effectors to VgrG proteins via binding at the VgrG C terminus (Schneider et al. 2013). It is therefore conceivable that one or more such proteins may bind to the C terminus of the *P. ananatis* LMG 2665^T VgrG proteins and recruit effectors. In this way, each complex, VgrG-1 or VgrG-2, together with their cognate effectors, might sit independently or alternatively at the tip of the TssD (Hcp) nanotube structures. Following contraction of the sheath, the T6SS-1 may thus be capable of delivering a multifunctional cargo or a multiple effector VgrG spike into the host cell in a single molecular translocation event. The effector proteins that may be secreted by the T6SS-1 of *P. ananatis* LMG 2665^T remains to be elucidated. Nevertheless, several genes of unknown function and limited or no conservation with other T6SSs are present in the *P. ananatis* LMG 2665^T T6SS-1 gene cluster. These genes may present candidates for system-specific effectors and will be studied in future.

P. ananatis LMG 2665^T was also found to exhibit antibacterial activity. On the basis of the 33 species tested in antibacterial competition assays, the host range was found to be restricted to selected Gram-negative bacteria. We subsequently investigated whether the T6SS-1 and

T6SS-3 contribute to the antibacterial activity of strain LMG 2665^T. The results indicate that strain LMG 2665^T does indeed require T6SS-1 but not T6SS-3 to inhibit competitor bacteria, since coculture with the wild-type or mutant 2665^{T}ΔT6SS-3 strains resulted in lower recovery of viable bacteria compared to the mutant 2665^{T}ΔT6SS-1 strain. As the antibacterial activity of the *P. ananatis* LMG 2665^T T6SS-1 is seen during coculture on solid agar medium surfaces, it may be that the T6SS-1 system acts through cell-cell contact (Dong et al. 2013; MacIntyre et al. 2010). We hypothesize that the antibacterial activity of *P. ananatis* LMG 2665^T could be mediated by T6SS-1-directed intoxication of other bacteria with protein effectors as part of a toxin-immunity system. By implication, strain LMG 2665^T must therefore itself possess a cognate immunity protein to prevent self-intoxication, whereas strains lacking cognate immunity proteins are inhibited. A functional link between T6SSs and toxin-immunity systems has been established in *P. aeruginosa* (Hood et al. 2010), *B. thailandensis* (Russell et al. 2012), *S. marcescens* (Chou et al. 2012; English et al. 2012) and *V. cholerae* (Dong et al. 2013). Bioinformatic analysis indicated that there are no obvious homologues to these toxins or immunity proteins in the genome of *P. ananatis* LMG 2665^T, thus supporting the notion that this bacterium may use a unique set(s) of effector and immunity proteins.

Recently, Rhs-family proteins of the soft-rot pathogen *Dickeya dadantii* were reported to mediate interbacterial competition (Koskinieni et al. 2013). Rhs proteins are characterized by sequence-diverse C-terminal regions and vary considerably between different strains of the same species. All *rhs* genes are closely linked to small downstream open reading frames that encode RhsI immunity proteins. These immunity proteins are also sequence-diverse and only protect against their cognate Rhs toxins (Koskinieni et al. 2013; Zhang et al. 2012). The Rhs proteins are secreted in a T6SS-dependent manner in both *S. marcescens* (Fritsch et al. 2013)

and *D. dadantii* (Koskinieni et al. 2013), suggesting that these proteins might constitute a new family of T6SS effectors. Interestingly, the genome of *P. ananatis* LMG 2665^T harbors two *rhs*/*rhsI* loci that are both located immediately adjacent to the T6SS-1 gene cluster only. Notably, one of the Rhs proteins, designated RhsD-1 (N454_00601), contains a conserved DUF4237 domain of unknown function at the C terminus. Given that Rhs toxins are encoded adjacent to theT6SS-1 gene cluster and that they appear to be secreted in a T6SS-dependent manner, it seems likely that one or both of the RhsD proteins may also play a role in *P. ananatis* LMG 2665^T pathogenesis. Indeed, RhsT from *P. aeruginosa* was shown to be translocated into phagocytic cells, where it induces inflammasome-mediated cell death (Kung et al. 2012). Moreover, *Salmonella* Typhimurium mutants lacking *rhs* were completely attenuated in pig and cattle models of infection (Chaudhuri et al. 2013). Taking all of the above into consideration, it is tempting to speculate that *P. ananatis* LMG 2665^T Rhs proteins are secreted in a T6SS-1-dependent manner and used as virulence factors against plant host cells, in addition to prokaryotes. Whether the Rhs-family proteins are indeed T6SS-1 effector proteins remains to be experimentally determined.

With the exception of *Salmonella* Typhimurium, it is noteworthy that the Gram-negative bacteria inhibited by *P. ananatis* LMG 2665^T are plant pathogens. *P. carotovorum* subsp. *carotovorum* is a pathogen of numerous vegetables, including, cucumber, onion, potato and cabbage (Toth et al. 2003), while *P. stewartii* subsp. *indologenes* has been isolated from symptomatic millet, pineapple and onion (Mergaert et al. 1993), and the *P. ananatis* strains LMG 2669 and LMG 2664 were both isolated from symptomatic pineapples displaying brown and grey rot (Spiegelber, 1958 – unpublished data). Notably, *Salmonella* Typhimurium, albeit not generally considered a plant pathogen, has been isolated from cantaloupe fruit (Gallegos-Robles et al. 2000) and is capable of internalizing tomato plants

(Gu et al. 2011). Considering that both these plants are hosts of *P. ananatis* (Countinho and Venter 2009), *Salmonella* Typhimurium may thus constitute a plausible competitor for *P. ananatis*. Based on the finding that *P. ananatis* LMG 2665^T uses its T6SS-1 not only for competition with other bacterial species, but also for competition within its own species, it is likely that killing of these bacteria in such a selective manner would be highly relevant to the ability of *P. ananatis* LMG 2665^T to mount a successful infection. The antibacterial T6SS-1 of strain LMG 2665^T could be used as a means of competitive exclusion, thereby creating a niche that is favorable for infection.

To determine whether the observed phenotypes are dependent on a functioning T6SS-1, we generated *tssA* and *tssD* (*hcp*) deletion mutants in the T6SS-1 gene cluster. These mutations led to attenuated pathogenesis in onion plants and antibacterial activity, while expression of TssA and TssD from a plasmid restored the wild-type strain LMG 2665^T phenotypes. The results indicate that the observed phenotypes were caused by T6SS-1 activity. As other studies have shown that TssD forms a nanotube that acts as a conduit to allow transport of T6SS-dependent effector proteins or protein complexes (Ballister et al. 2008; Leiman et al. 2009; Schneider et al. 2013), it is likely that deletion of *tssD* in *P. ananatis* LMG 2665^T causes defects in the assembly of the secretion apparatus or in the secretion of T6SS-1-dependent effectors, either of which would likely compromise T6SS-1 functionality. Although the function of the cytoplasmic TssA protein is not known (Cascales and Cambillau 2012), it has been proposed that TssA may play a regulatory role (Shrivastava and Monde 2008). More recently, TssA was shown to interact with TssK, a cytoplasmic protein that is responsible for TssB-TssC sheath polymerization (Zoued et al. 2013). TssA may thus indirectly regulate the assembly of TssB-TssC tubules through its interaction with TssK, but this requires further investigation.

In conclusion, we report the presence of a functional T6SS (T6SS-1) in *P. ananatis* LMG 2665^T and provide evidence assigning functions to this T6SS in pathogenesis and bacterial competition. Further studies are needed to identify genes involved in the assembly and the mechanism of secretion of the T6SS-1, including the detection of TssD (Hcp) secretion, as well as the nature of any effectors or toxins. This study has added to our understanding of how *P. ananatis* causes disease, and provides a new potential target for control of diseases caused by this plant pathogen.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions.

The bacterial strains and plasmids used in this study are listed in Table 1. All *P. ananatis* strains in this study were derived from wild-type strain LMG 2665^T. Bacterial strains were grown on LB agar or in LB broth at 32°C (*P. ananatis*) or 37°C (*E. coli*) with shaking at 250 rpm. For plasmid DNA selection and maintenance, the growth medium was supplemented with kanamycin (50 μg/ml), gentamycin (20 μg/ml) or chloramphenicol (50 μg/ml). The growth of *P. ananatis* wild-type and mutant strains was compared in LB broth. To prepare inoculum for plant inoculations, *P. ananatis* cultures grown overnight in LB broth were diluted 100-fold in fresh LB broth and the cultures were incubated until they reached an optical density at 600 nm (OD_{600}) of 0.4. The cells were harvested by centrifugation (7000 × *g*, 3 min, 4°C) and suspended in sterile distilled water (dH_2O) until an OD_{600} of 0.1 (approximately 6.2×10^6 CFU/ml as determined by dilution plating).

***In planta* growth curve assays.**

The growth of *P. ananatis* wild-type and mutant strains was compared in onion (*Allium cepa* cv. Texas grando), which in previous studies have been shown to be an excellent experimental host for *P. ananatis* (Goszczynska et al. 2006; Morohoshi et al. 2007). To enable enumeration of the bacteria, the respective strains were electroporated with plasmid pMP7605 to confer gentamycin resistance prior to the preparation of inoculum as described above. Leaves of six-week-old onion seedlings were used for inoculation. Each leaf was inoculated with 3 µl of a standardized bacterial suspension (6.2×10^6 CFU/ml) and the plants were incubated in a greenhouse at 28°C. Leaves were collected at 10 to 72 h postinoculation, macerated in 2 ml of TE buffer (10 mM Tris-Cl, 1 mM EDTA; pH 8.0), serially diluted in sterile dH_2O and then plated in duplicate on LB agar supplemented with gentamycin.

Recombinant DNA techniques.

Molecular cloning techniques used in the construction of recombinant plasmids were carried out using standard procedures (Sambrook and Russell 2001). Restriction enzymes, calf intestine alkaline phosphatase, Klenow fragment of *E. coli* DNA polymerase I and T4 DNA ligase (Roche Diagnostics, Mannheim, Germany) were used according to the manufacturer's protocols. Plasmid DNA was extracted from *E. coli* with a Zyppy Plasmid Miniprep kit, genomic DNA was isolated from *P. ananatis* strains with a Quick gDNA isolation kit, and restriction DNA fragments were purified from agarose gels by use of a Zymoclean Gel DNA Recovery kit (all kits obtained from Zymo Research Corp., Orange, CA, U.S.A.). Plasmid constructions were first established in *E. coli* DH5α and then transferred to *P. ananatis* strains. Electro-competent cells were prepared and transformed according to published procedures for *E. coli* (Cohen et al. 1972) and *P. ananatis* (Katashkina et al. 2009). PCR assays were performed with SuperTherm DNA polymerase (Whitehead Scientific, Cape

Town, South Africa) and PCR amplicons were purified with the DNA Clean and Concentrator kit (Zymo Research Corp.). Primers used in this study were designed from the *P. ananatis* LMG 2665^T genome sequence and obtained from Inqaba Biotechnical Industries (Pretoria, South Africa). Southern blot hybridization was performed using the DIG-High Prime DNA labeling and detection starter kit (Roche Diagnostics). Nucleotide sequencing was performed with the ABI PRISM BigDye terminator v.3.1 cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA, U.S.A.), followed by resolution on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems), in accordance with the manufacturer's instructions. All plasmid constructs were verified by restriction endonuclease digestion and by nucleotide sequencing.

Generation of *P. ananatis* LMG 2665^T mutant strains.

Mutant strains with deletions of T6SS-1 (*N454_00602* to *N454_00638*), T6SS-3 (*N454_0002* to *N454_0008*), *tssA* (*N454_00632*) or *tssD* (*N454_00629*; *hcp*) were constructed with the lambda Red-recombineering method as described previously (Datsenko and Wanner 2000; Katashkina et al. 2009). Gene cluster and single gene disruption cassettes were first constructed by an overlap-extension PCR protocol (Shevchuk et al. 2004). Briefly, 400- to 900-bp DNA fragments contiguous to the 5' and 3' ends of targeted genes were PCR amplified by using LMG 2665^T chromosomal DNA template and the appropriate F_{up}/R_{up}-kan and F_{down}-kan/R_{down} primers (Table S1, Fig. S1). Each of the R_{up}-kan and F_{down}-kan primers contained 20 nucleotides that are homologous to the 5' and 3' termini of a kanamycin resistance gene, respectively. Plasmid pkD13 was used as template to amplify the kanamycin resistance gene with flanking regions homologous to the target gene using the appropriate F-Kan and R-Kan primers (Table S1). In a second PCR, 20 ng of each of the purified amplified

DNA fragments were mixed and the fused DNA fragments were obtained by overlap-extension PCR with the appropriate F_{up}/R_{down} primers.

To enable lambda Red-dependent integration of the fused DNA fragments, the purified disruption cassettes were introduced into electro-competent *P. ananatis* LMG 2665^T carrying the plasmid pRSFRedTER, which expresses the lambda-Red recombinase system and encodes a *sacB* counter-selection gene (Katashkina et al. 2009). An overnight culture of *P. ananatis* LMG 2665^T(pRSFRedTER) was diluted 100-fold in LB broth supplemented with chloramphenicol and 1.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) to induce expression of the lambda recombinase system. The culture was grown to an OD_{600} of 0.5 at 32°C. Bacteria were then made electro-competent and transformed with 300-500 ng of the corresponding purified PCR-generated disruption cassettes. The resulting strains were selected by kanamycin resistance on LB agar. Selected strains were cured from the pRSFRedTER plasmid DNA by streaking on LB agar containing 10% (w/v) sucrose and loss of the plasmid DNA was confirmed by streaking the strains on agar containing chloramphenicol. Allelic replacement in mutant strains that were chloramphenicol-sensitive but kanamycin-resistant was confirmed by PCR with primers flanking the deletion sites (F_{up}-out/R_{down}-out) and sequencing of the amplicons, as well as by Southern blot hybridization (data not shown).

Complementation of *P. ananatis* LMG 2665^T Δ*tssA* and Δ*tssD* knockout mutants.

To complement the *tssA* and *tssD* genes, the full-length *tssA* (1.023 kb) and *tssD* (483 bp) genes, together with upstream regions to include putative promoters (up to 540 bp), were PCR amplified using *P. ananatis* LMG 2665^T genomic DNA as template and the appropriate primers (Table S1). The amplicons were treated with Klenow polymerase, blunt-end cloned

into the promoterless broad-host-range cloning vector pBRRMCS-5 and then transformed into *E. coli* DH5α. The complementation plasmids pBRR-*tssA* and pBRR-*tssD* were extracted from *E. coli*, electroporated into the corresponding *P. ananatis* mutant strains and complemented strains were selected by gentamycin resistance on LB agar.

Pathogenicity assays.

Pathogenicity of *P. ananatis* wild-type and mutant strains was determined in onion plants as described previously (Goszczynska et al. 2006; Morohoshi et al. 2007). In each test, at least four leaves of six-week-old onion plants (*Allium cepa* cv. Texas grando) were inoculated with 3 µl of the inoculum (6.2×10^6 CFU/ml) under the epidermis of the leaf. Sterile dH_2O was included in the assay as a negative control. On average, 20 onion plants were inoculated per strain and each experiment was repeated three independent times. The plants were maintained in the greenhouse during the evaluation period at a temperature of 25 to 28°C, and natural day and night cycles. Plants were visually inspected daily for development of disease symptoms. At three days postinoculation, the number of inoculated wilted (dead) leaves per plant for each strain was recorded and the average percentage of dead leaves in all three biological repeats was calculated.

Bacterial competition assays.

Bacterial strains used for the competition studies are provided in Table S2. The competition assays were carried out with a slightly modified version of a protocol described by MacIntyre et al. (2010). To ensure selection of competitor cells for counting, each of the bacterial strains was transformed with plasmid pMP7605 to confer gentamycin resistance. For competition assays, each strain was grown overnight in LB broth supplemented with antibiotic, and then collected and washed twice in sterile LB broth (7000 × *g*, 2 min). The cell suspensions were

normalized to an OD_{600} of 0.1 and mixed at a ratio of 1:1 with either the *P. ananatis* wild-type or mutant strains. Twenty µl of the mixture was spotted on LB agar and incubated overnight at 32°C for all targeted bacteria analyzed, excepting *Pectobacterium* spp., which were cocultured with *P. ananatis* at 28°C. After incubation, the bacteria were recovered from the agar plates and suspended in 1 ml of sterile LB broth, and serial dilutions were plated in duplicate on LB agar supplemented with gentamycin.

Statistical analyses.

Statistical significance of the data obtained in bacterial competition assays was evaluated by making use of an unpaired, two-tailed Student's *t*-test using JMP software (v.5; SAS Institute Inc., Cary, NC, U.S.A.). *P* values of less than 0.05 were considered to be significant.

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AUTHOR CONTRIBUTIONS

All of the authors participated in conceiving and designing the experiments, analysis and interpretation of the data, and drafting of the manuscript. The experiments were performed by DYS.

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Supplementary material

Table S1. Primers used in this study

Primer	Sequence (5' to 3')	Use
T6SS-1 primers		
C1-F_{up}	CCAGATATTGCGGTGCGCTGTG	Gene disruption cassette
C1-R_{up}-kan	AGCTCCAGCCTACACAATCGCAGATGGGCCATATTCAGCAG	Gene disruption cassette
C1-F_{down}-kan	GGTCCGACGGATCCCCGGAATTGCTCACCATTGTGTCATCAG	Gene disruption cassette
C1-R_{down}	GACTTAATTACGATCTCAGAC	Gene disruption cassette
C1-F-Kan	CTGCTGAATATGGCCCATCTGCGATTGTGTAGGCTGGAGCT	Amplification of Km^r gene
C1-R-Kan	CTGATGACACAATGGTGAGCAATTCCGGGGATCCGTCGACC	Amplification of Km^r gene
C1-F_{up}-out	AGCATCAAGATCATAATGCATG	Verification of deletion
C1-R_{down}-out	GCATGTTCCTCGACTGGACAG	Verification of deletion
T6SS-3 primers		
C3-F_{up}	TGAATGTTGAACGTCACAGAGC	Gene disruption cassette
C3-R_{up}-kan	AGCTCCAGCTACACAATCGCCGTAACGACGAGCAGAATAAC	Gene disruption cassette
C3-F_{down}-kan	GGTCGACGGATCCCCGGAATTTGTCAGCAGGAATAATCACC	Gene disruption cassette
C3-R_{down}	ACACCCTGGTCACCAGTGATGTG	Gene disruption cassette
C3-F-Kan	GTTATTCTGCTCGTCGTTACGGCGATTGTGTAGGCTGGAGCT	Amplification of Km^r gene
C3-R-Kan	GGTGATTATTCCTGCTGACAAATTCCGGGGATCCGTCGACC	Amplification of Km^r gene
C3-F_{up}-out	AGCGAAGCGATCTTCCGGAGC	Verification of deletion
C3-R_{down}-out	AAGTCTTAGGTAGACTGAGCG	Verification of deletion
***tssA* primers**		
tssA-F_{up} [a]	AAGCAGGCTGTTTAGTGGATCG	Gene disruption cassette
tssA-R_{up}-kan	AGCTCCAGCCTACACAATCGCGACTAAGCTAACAGTCAAG	Gene disruption cassette
tssA-F_{down}-kan	GGTCGACGGATCCCCGGAATCATCCGCTTCGCCTCAGG	Gene disruption cassette
tssA-R_{down} [a]	TGGTACCACCGATGTTGGGTAC	Gene disruption cassette
tssA-F-Kan	CTTGACTGTTAGCTTAGTCGCGATTGTGTAGGCTGGAGCT	Amplification of Km^r gene
tssA-R-Kan	CCTGAGGCGAAGCGGATGATTCCGGGGATCCGTCGACC	Amplification of Km^r gene
tssA-F_{up}-out	TGGTTGTTGAGAAGTCTGGTTC	Verification of deletion
tssA-R_{down}-out	TCCAGCAGGCGCAGTCGATTCG	Verification of deletion
***tssD* primers**		
tssD-F_{up} [b]	ATTAAGTAGAACTTCTAATTCATTG	Gene disruption cassette
tssD-R_{up}-kan	AGCTCCAGCCTACACAATCGCCTGGCTGGAATATCAAAGAG	Gene disruption cassette
tssD-F_{down}-kan	GGTCGACGGATCCCCGGAATCCTTCAAATACATATCAATAGC	Gene disruption cassette
tssD-R_{down} [b]	TGCGAGCAACTGCACTAAAGCATG	Gene disruption cassette
tssD-F-Kan	CTCTTTGATATTCCAGCCAGGCGATTGTGTAGGCTGGAGCT	Amplification of Km^r gene
tssA-R-Kan	CAAATACATATCAATAGCATTCCGGGGATCCGTCGACC	Amplification of Km^r gene
tssD-F_{up}-out	CTTATCCATGATTAAGTCTACAGC	Verification of deletion
tssD-R_{down}-out	CTGCGGAGTACCACGATGCTGAC	Verification of deletion
Other primers		
Km1	CAGTCATAGCCGAATAGCCT	Verification of mutant
Km2	CGGTGCCCTGAATGAACTGC	Verification of mutant

[a] Primers used to amplify the *tssA* gene for complementation plasmid construction.

[b] Primers used to amplify the *tssD* gene for complementation plasmid construction.

Km^R = kanamycin resistance gene.

Table S2. Bacterial strains used as competitors in bacterial competition assays

Bacterial strains[a]	Relevant characteristics or host of isolation	Source
Gram-positive		
Bacillus cereus Mn106-2a2c	Isolated from termite	FABI[b]
Bacillus subtilis A	Environmental isolate	FABI
B. subtilis B	Environmental isolate	FABI
Gram-negative		
Escherichia coli DH5α	Derivative of *E. coli* K12	Invitrogen
Pantoea ananatis LMG 2669	Pathogen of pineapple	FABI
P. ananatis LMG 2664	Pathogen of pineapple	FABI
P. ananatis AJ13355	Isolated from soil in Japan	FABI
P. ananatis BD442	Pathogen of maize	FABI
P. ananatis PA-4	Pathogen of onion	FABI
P. ananatis ICMP 10132	Pathogen of sugarcane	FABI
P. ananatis ATCC 35400	Pathogen of honeydew melon	FABI
P. ananatis LMG 2678	Pathogen of wheat	FABI
P. ananatis LMG 2101	Pathogen of rice	FABI
P. ananatis LMG 20104	Pathogen of *Eucalyptus* sp.	FABI
P. ananatis Uruguay 40	Pathogen of *Eucalyptus* sp.	FABI
P. ananatis BD 301	Pathogen of onion	FABI
P. ananatis BD 622	Pathogen of maize	FABI
P. ananatis Mmir 9	Isolated from *Mirridiae* sp.	FABI
P. vagans BCC006	Isolated from *Eucalyptus grandis*	FABI
P. eucalypti LMG 24197^T	Isolated from *Eucalyptus grandis*	FABI
P. stewartii subsp. *indologens*	Pathogen of maize	FABI
Pectobacterium atrosepticum LMG 6687	Pathogen of tomato	FABI
Pectobacterium betavasculorum LMG 2398	Pathogen of potato	FABI
Pectobacterium carotovorum subsp. *carotovorum* LMG 2404^T	Pathogen of Irish potato	FABI
Pectobacterium carotovorum subsp. *brasiliensis* 1692	Pathogen of potato	FABI
Brenneria nigrifluens LMG 2696	Pathogen of walnut	FABI
Brenneria quercina LMG 5952	Pathogen of oak	FABI
Salmonella enterica serovar Typhimurium	Clinical isolate	FABI
Klebsiella pneumonia TMA5	Isolated from *Eucalyptus* sp. in Thailand	FABI
Serratia marcescens LMG 2792^T	Isolated from pond water	FABI
Burkolderia sp. P19	Isolated from palm tree	FABI
Pseudomonas putida WRB111	Isolated from *Eucalyptus* sp.	FABI
Enterobacter sakazakii M658	Isolated from milk powder in the UK	FABI

[a] Each bacterial strain was transformed with plasmid pMP7605, which confers gentamycin resistance, prior to use in competition assays with *Pantoea ananatis* LMG 2665^T and derived isogenic mutant strains.

[b] FABI, Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa.

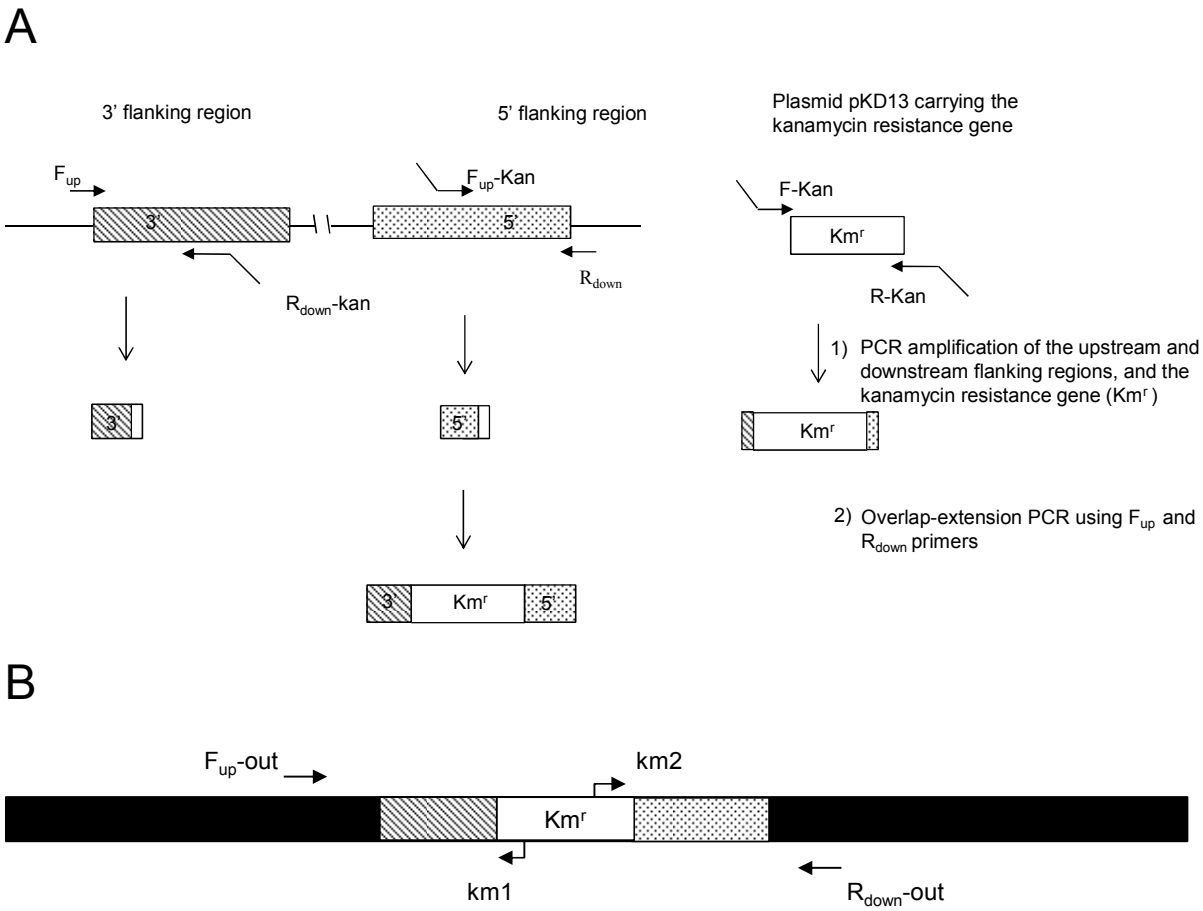

Fig. S1. Summary of the PCR reactions required for (**A**) construction of a gene disruption cassette and (**B**) verification of newly introduced deletions. The first step is to PCR amplify the upstream and downstream sequences flanking the targeted gene cluster or individual genes, and the kanamycin resistance gene that is used as a selectable marker. The PCR products are then fused in an overlap-extension PCR to generate the desired gene disruption cassette. Deletion of the targeted gene clusters or individual genes in the constructed mutant strains were screened for by making use of the primer pairs indicated in figure B.