Ralstonia solanacearum needs Flp pili for virulence on potato

Charles K. Wairuri[1], Jacquie E. van der Waals[1], Antoinette van Schalkwyk[2], and Jacques Theron[1*]

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

[2]Inqaba Biotechnical Industries, P.O. Box 14356, Pretoria, South Africa

*Corresponding Author: Prof J. Theron

Department of Microbiology and Plant Pathology

University of Pretoria

Pretoria 0002

South Africa

E-mail: jacques.theron@up.ac.za

Tel: +27 12 420-2994

Fax: +27 12 420-3266

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ABSTRACT

Type IV pili are virulence factors in various bacteria. Several subclasses of type IV pili have been described according to the characteristics of the structural prepilin subunit. Although type IVa pili have been implicated in the virulence of *Ralstonia solanacearum*, type IVb pili have not previously been described in this plant pathogen. Here, we report the characterization of two distinct *tad* loci in the *R. solanacearum* genome. The *tad* genes encode functions necessary for biogenesis of the Flp subfamily of type IVb pili initially described for the periodontal pathogen *Aggregatibacter actinomycetemcomitans*. To determine the role of the *tad* loci in *R. solanacearum* virulence, we mutated the *tadA2* gene located in the megaplasmid that encodes a predicted NTPase previously reported to function as the energizer for Flp pilus biogenesis. Characterization of the *tadA2* mutant revealed that it was not growth impaired *in vitro* or *in planta*, produced wild-type levels of EPS galactosamine, and exhibited swimming and twitching motility comparable to the wild-type strain. However, the *tadA2* mutant was impaired in its ability to cause wilting of potato plants. This is the first report where type IVb pili in a phytopathogenic bacterium contribute significantly to plant pathogenesis.

INTRODUCTION

Bacteria can assemble various cell surface appendages that enable them to colonize diverse biotic and abiotic surfaces. Indeed, the attachment of a pathogenic bacterium to the surface of a eukaryotic cell is an important step for successful infection, and also a prerequisite for subsequent events such as internalization and contact-dependent translocation of effector molecules by type III secretion systems (Henderson et al. 1999; Buttner and Bonas 2002). For interactions with surfaces, many bacteria make use of fimbrial or non-fimbrial adhesins

(Danhorn and Faqua 2007; Gerlach and Hensel 2007). Fimbriae or pili are hair-like structures that radiate from the bacterial surface and typically consist of only one structural protein called pilin (Pelicic 2008). Although gram-negative bacteria produce various different pili (Fronzes et al. 2008), the type IV pili (T4P) are the most abundant pili described thus far. The functions of T4P are quite diverse and they have been shown to play an important role in adhesion of pathogenic bacteria to their host cells, biofilm formation, twitching motility, conjugative DNA transfer and bacteriophage infection (Mattick et al. 1996; O'Toole and Kolter 1998; Wall and Kaiser 1999; Kang et al. 2002; Craig and Li 2008). Therefore, it is not surprising that pili are essential virulence factors of many bacteria.

Based on sequence similarities, T4P can be divided into type IVa (T4a) and type IVb (T4b) pili. The N-terminal signal sequence of the T4a prepilin is relatively short and consists of 5 to 6 amino acids (aa), while the signal sequence of T4b prepilin contains 15 to 30 aa. Mature T4a pilins have a characteristic length of 150-160 aa, whereas T4b pilins are either long (180-200 aa) or short (40-50 aa) (Pelicic 2008). Studies on *Aggregatibacter actinomycetemcomitans*, a gram-negative bacterium responsible for localized juvenile periodontitis, revealed the presence of T4b pili with specific characteristics. These include a signal sequence of variable length (10 to 26 aa), a relatively short mature pilin (smaller than 90 aa) and a shared "Flp" motif of 20 hydrophobic residues at the N-terminus of the mature pilin, with adjacent glutamate and tyrosine residues in its centre (Kachlany et al. 2001; Planet et al. 2003). Due to its unique features, it has been described as the Flp subfamily of T4b pili (Kachlany et al. 2001). In *A. actinomycetemcomitans*, the Flp pilin requires for its assembly a subset of components, called Tad and Rcp, which includes the RcpA secretin (Clock et al. 2008), the TadD pilotin or docking protein (Clock et al. 2008), the TadA trafficking NTPase (Bhattacharjee et al. 2001), the PilC-like proteins TadB and TadC (Wang and Chen 2005),

the TadE and TadF pseudopilins (Kachlany et al. 2000), and the TadV prepilin peptidase (Perez et al. 2006; Tomich et al. 2006). The genes of other Tad system components (RcpB, RcpC, TadG and TadZ) do not show extensive homologies to any genes and have not been assigned predicted functions.

Similar *tad* loci to that described in *A. actinomycetemcomitans* have been identified in a variety of bacterial and archaeal species, usually as a single copy but in up to four copies in some species (Kachlany et al. 2000; Planet et al. 2003; Tomich et al. 2007). Comparative phylogenetic analysis suggests that many bacterial species have acquired *tad* genes from foreign sources, and because of its apparent propensity for horizontal gene transfer, the *tad* locus has also been named a widespread colonization island (Planet et al. 2003). The *tad* locus has been implicated in the pathogenesis of several bacteria, including *A. actinomycetemcomitans* (Schreiner et al. 2003), *Haemophilus ducreyi* (Nika et al. 2002; Spinola et al. 2003), *Yersinia ruckeri* (Fernandez et al. 2004) and *Pasteurella multocida* (Fuller et al. 2000). In contrast to these human and animal pathogenic bacteria, the functional significance of the *tad* locus in phytopathogenic bacteria has not yet been reported.

Ralstonia solanacearum is a soilborne gram-negative bacterium that causes bacterial wilt disease in more than 200 plant species, representing over 50 botanical families (Hayward 1991; Swanson et al. 2005). Amongst these, solanaceous plants, including economically significant hosts of global importance, such as potato, tomato, peanut and eggplants are the most affected species (Janse et al. 2004). Due to its wide geographic distribution and unusually broad host range the pathogen is responsible for severe crop losses worldwide. *R. solanacearum* normally invades plant roots through wounds or where secondary roots emerge, penetrates the xylem and then systematically colonizes the vascular system.

Extensive colonization disrupts vascular function and the plants rapidly wilt and die (Schell 2000; Vasse et al. 2005). *R. solanacearum* possesses various virulence factors that act quantitatively to cause disease. In addition to extracellular polysaccharide I (Denny and Baek 1991; McGarvey et al. 1999), cell wall-degrading enzymes (Gonzalez and Allen 2003; Liu et al. 2005), and type III-secreted effectors (Cunnac *et al*. 2004), flagellar-driven swimming and pilus-driven twitching motility are also necessary for virulence (Tans-Kersten et al. 2001; Liu et al. 2001). All of these virulence factors are controlled by a complex regulatory signal transduction pathway (Schell 2000; Hikichi et al. 2007) that responds to both environmental signals and quorum sensing (Brito et al. 1999). Although much is understood about these virulence factors and their regulation, less is known about how *R. solanacearum* effectively adheres, colonizes and spreads in the host.

To enrich our knowledge of the gene machinery required by *R. solanacearum* to cause disease in host plants, we mined the complete genome sequence of *R. solanacearum* GMI1000 (Salanoubat et al. 2002) and identified a number of genes homologous to the *flp-rcp-tad* genes previously characterized in *A. actinomycetemcomitans* (Kachlany et al. 2000). We made use of a targeted mutagenesis strategy, coupled with genetic complementation analysis, to evaluate the role of Flp pili in *R. solanacearum* virulence and other properties. We report that a mutant strain, derived from mutagenesis of the *R. solanacearum tadA2* gene in the megaplasmid *tad* locus, displayed significantly reduced virulence on potato plants.

RESULTS

Characterization of *R. solanacearum tad* gene clusters

Mining of the *R. solanacearum* GMI1000 genome identified two distinct *tad*-like gene clusters present in the megaplasmid and chromosome, respectively, which each harbor genes with similarity to the *flp-rcp-tad* genes previously identified in *A. actinomycetemcomitans* (Kachlany et al. 2000). Indeed, most of the genes of the *tad* locus from *A. actinomycetemcomitans* can be identified in a similar genetic organization in the respective *tad* loci of *R. solanacearum* (Fig. 1A). The transcriptional organization of the *R. solanacearum tad* loci is, however, not yet known.

The names and characteristics attributed to the RSp1079-to-RSp1092 ORFs that comprise the putative *tad* locus in the *R. solanacearum* GMI1000 megaplasmid are provided in Table 2. The RSp1092 ORF was identified as an *flp*-like gene (Kachlany et al. 2001; Perez et al. 2006). The putative Flp precursor displays features characteristic of the Flp subfamily of T4b pili (Fig. 1B). As indicated earlier, these include a relatively short mature pilin (44 aa) and a Flp motif at the N-terminus of the mature pilin. In addition, it contains a phenylalanine (Phe) residue towards its C-terminus that is conserved in most predicted Flp proteins (Kachlany et al. 2001). The megaplasmid *tad* locus, however, lacks homologues of the *flp-2*, *rcpB* and *tadE* genes, which are found in the *A. actinomycetemcomitans flp-rcp-tad* gene cluster. Notably, *rcpB* has so far been identified only in the *tad* loci of *Pasteurellaceae* (Perez et al. 2006), whereas *flp-2*, in contrast to *flp-1*, is not required for the production of Flp pili or for adherence of *A. actinomycetemcomitans* (Perez et al. 2006). Moreover, the *tad* locus of several bacteria encodes only a single pseudopilin protein (TadE or TadF) (Tomich et al. 2007), which in the case of *Pseudomonas aeruginosa* has been reported to be dispensable for

Flp biogenesis (Bernard et al. 2009). No obvious homology could be found between the putative proteins encoded by RSp1079, RSp1081 and RSp1088 and the Flp-Tad-Rcp system of *A. actinomycetemcomitans*. However, RSp1081 encodes a putative homologue of PA4298, which has thus far been reported only in the *tad* locus of *P. aeruginosa*, but its function is not yet known (de Bentzmann et al. 2006).

The *R. solanacearum* GMI1000 chromosomal *tad* locus also contains 14 ORFs (RSc0648 through RSc0661), but it lacks homologues to three ORFs in the megaplasmid *tad* locus (*i.e.* RSp1079, RSp1081 and RSp1088) and encodes four putative Flp prepilin proteins. The Flp putative proteins all lack a Phe residue at the C-terminus, while the putative protein encoded by RSc0659 lacks the Flp motif (Fig. 1B). In addition to lacking homologues of *rcpB* and *tadF*, no obvious homologue of the *A. actinomycetemcomitans tadD* gene could be identified in the chromosomal *tad* locus. The TadD protein of *A. actinomycetemcomitans* is localized to the bacterial outer membrane and has been reported to be critical for the assembly, transport and functioning of the secretion complex (Tomich et al. 2007; Clock et al. 2008). Moreover, during *in vivo* assays to identify virulence genes, *tadD* was identified as essential for virulence in *P. multocida* and *Y. ruckeri* (Fuller et al. 2000; Fernandez et al. 2004). Finally, no obvious homology could be found between the putative protein encoded by the RSc0654 ORF and the Flp-Tad-Rcp system of *A. actinomycetemcomitans*. The ORFs comprising the chromosomal *tad* locus have been renamed in accordance with the Flp-Rcp-Tad nomenclature (Table 2).

Considering the genetic variability among *R. solanacearum* strains, it was of interest to determine whether the *tad* loci also occur in strains other than the GMI1000 strain. Analysis of the genome sequences of five *R. solanacearum* strains (CMR15, PSI07, CFBP2957,

IPO1609 and Po82) indicated the presence of similar *tad* loci in the megaplasmid and chromosome of these strains (Supplementary material: Fig. 1). Compared to the *R. solanacearum* GMI1000 megaplasmid *tad* locus, all five strains analyzed lacked a homologue of RSp1088, whereas strain Po82 also lacked a homologue of RSp1081. Comparison of the chromosomal *tad* loci indicated the presence of homologues to each of the ORFs in the *R. solanacearum* GMI1000 chromosomal *tad* locus, but the strains differed with regards to the number of putative *flp* alleles and ranged between two and five clustered *flp* alleles (Supplementary material: Fig. 1). These results suggest that the presence of *tad* loci may be common among *R. solanacearum* strains.

Construction of *R. solanacearum* NB336-1085 mutant

Although the genome sequence of *R. solanacearum* NB336 is not known, it is likely that this strain, like other *R. solanacearum* strains, also harbors two *tad* loci. PCR amplification and sequencing revealed that the coding regions of the *tadA1* (GenBank accession no. JN998027) and *tadA2* (GenBank accession no. JN968967) genes of NB336 were similar to those of *R. solanacearum* GMI1000 (95% and 99% amino acid sequence similarity, respectively) (data not shown). To generate a *R. solanacearum* mutant strain that would be useful in subsequent studies, we introduced a targeted mutation into the putative *tadA2* gene present in the megaplasmid *tad* locus of *R. solanacearum* NB336. This was based on the following considerations: (i) most of the virulence genes of *R. solanacearum* are reported to be located in the megaplasmid (Salanoubat et al. 2002; Genin and Boucher 2004); (ii) the megaplasmid *tad* locus contains a TadD homologue, which has been implicated as being required for proper secretin assembly and function (Clock et al. 2008); (iii) the *tadA* gene is highly conserved in the *tad* loci of different bacteria (Planet et al. 2001; Tomich et al. 2007); (iv) the *tadA* gene encodes an NTPase essential for energizing the assembly or secretion of Flp pili in

A. actinomycetemcomitans (Bhattarcharjee et al. 2001); (v) inactivation of the TadA proteins of *A. actinomycetemcomitans* and *H. ducreyi* results in bacterial strains attenuated for virulence (Schreiner et al. 2003; Spinola et al. 2003); and (vi) *tadA* mutants of human pathogenic bacteria lack Flp pili on the surface of the cells (Bhattarcharjee et al. 2001; Nika et al. 2002; de Bentzmann et al. 2006).

We generated a mutant *tadA2* allele by excising an internal fragment of the subcloned *tadA2* ORF, thus deleting the conserved Asp and His boxes and the Walker B motif that are all essential for NTPase functioning (Whitchurch et al. 1991; Possot and Pugsly 1994), and replacing it with a gentamycin resistance cassette. The mutagenesis construct was then introduced into the megaplasmid of the wild-type strain NB336 by allelic replacement to generate the mutant strain NB336-1085. The wild-type and mutant *R. solanacearum* strains were subsequently examined for the presence of potential Flp pili. Transmission electron microscopy (TEM) revealed differences between the wild-type and mutant *R. solanacearum* cells with regard to the cell-surface appendages present (Fig. 2). Negative staining of the wild-type NB336 strain revealed the presence of flagella, pili and darkly stained thick bundled pili structures. In contrast, the latter exostructures were not observed in the NB336-1085 mutant strain. The bundled pili, referred to as Flp pili, could be differentiated readily from other pili. They were less abundant, stained darker and comprised bundles of long thin pili. Complementation of the mutant strain with a copy of the wild-type *tadA2* gene *in trans* restored the wild-type phenotype, albeit that the long bundled fibrils were thinner and appeared less rigid.

Phenotypic characterization of *R. solanacearum* NB336-1085

To determine whether mutagenesis had unintended side-effects, we compared the *R. solanacearum* strains for traits known to affect virulence such as elicitation of a hypersensitive reaction (HR), EPS galactosamine production and motility. The results of these phenotypic assays are provided in the supplementary material (Supplementary material: Figs. 2 to 4). The NB336-1085 mutant strain had mucoid colony morphology on TZC agar and grew as well as the wild-type NB336 strain in CPG broth (data not shown) and *in planta* (Fig. 3). The NB336-1085 mutant elicited a normal wild-type HR defense response when infiltrated into non-host tobacco leaves, indicating that Hrp pili and the rest of the type III secretion system was not affected by the introduction of the mutant allele. Galactosamine is a major constituent of the acidic EPS I polymer (Denny et al. 1988; Araud-Razou et al. 1998) and considered to be a reliable indicator of the total EPS produced (Brumbley and Denny 1990). Quantification of the EPS galactosamine produced by the *R. solanacearum* strains indicated that that they produced similar amounts of EPS galactosamine and therefore the membrane-localized functions for EPS I biosynthesis were not affected in the mutant strain.

On motility agar, colonies of the *R. solanacearum* strains were surrounded by an even white halo, indicative of flagellum-driven motility, and the motility halo of each strain was similar in size. To assess twitching motility, the bacterial colonies on CPG agar were compared under a light microscope. Individual rafts of cells with jagged edges were observed, indicating that they were the result of cells migrating over the agar surface rather than being due to multiplication away from the centre of the colony. The NB336-1085 mutant strain thus displayed swimming and twitching motility comparable to that of the wild-type NB336 strain, indicating normal production of flagella and T4a pili.

***R. solanacearum* NB336-1085 displays reduced virulence**

To evaluate the virulence of the NB336-1085 mutant strain, we performed two types of virulence assay on a susceptible potato host. In the naturalistic soil soak assay, which requires bacteria to locate and invade host roots from the soil (Tans-Kersten et al. 2001), wild-type NB336 caused a disease index of 3.94 at 16 days after inoculation and 4 at 30 days after inoculation. In contrast, the NB336-1085 mutant strain did not cause wilting over the 30-day period. Notably, the complemented mutant strain NB336-1085comp restored the virulence phenotype. Although the NB336-1085comp strain caused a disease index of 4 at 29 days, it was significantly (P = 0.05) slower than the wild-type strain in causing wilting according to Tukey's HSD test performed on each days 8-23 (Fig. 4A). In a cut-petiole assay, which bypasses the normal infection route and introduces bacteria directly into the vascular system (Liu et al. 2001), the wild-type NB336 strain wilted all potato plants by day 12 after inoculation. The NB336-1085comp strain wilted all potato plants by day 16 and was statistically indistinguishable from the wild-type strain in this assay. However, potato plants inoculated with the NB336-1085 mutant strain did not wilt (Fig. 4B) and developed only restricted leaf yellowing and localized necrosis (Fig. 4C). In both these assays, the complemented mutant strain induced wilting at a lower rate than the wild-type NB336 strain, possibly because of differences in the *tadA2* expression level due to copy number or promoter effects. These results nevertheless suggested that the megaplasmid *tad* locus of *R. solanacearum*, or functions that require the *tadA2*-encoded protein, is important for virulence on potato plants.

Biofilm formation is affected on an abiotic surface

Many plant-associated bacteria, including *R. solanacearum*, form biofilms in contact with biotic or abiotic environments (Morris and Monier 2003; Danhorn and Fuqua 2007). Since Flp pili have been shown to mediate biofilm development (Kachlany et al. 2000; Nika et al. 2002; de Bentzmann et al. 2006), we sought to characterize biofilm formation of the wild-type and mutant strains by making use of quantitative PVC microtiter plate assays. After the *R. solanacearum* strains were incubated for 48 h at 30°C, biofilm bands were observed at the air-liquid interface. Interestingly, the NB336-1085 mutant strain formed significantly more biofilm than the wild-type NB336 strain. However, strain NB336-1085comp, in which the mutation was complemented *in trans*, produced a similar amount of biofilm compared to the wild-type NB336 strain (Fig. 5). These results indicated that the ability of the NB336-1085 mutant strain to form biofilms was significantly enhanced under the tested conditions.

DISCUSSION

Bioinformatic analyses revealed the presence of two distinct 14-gene *tad* loci, located in the chromosome and megaplasmid of *R. solanacearum* GMI1000, and similar *tad* loci were also identified in the sequenced genomes of other *R. solanacearum* strains. The *tad* genes encode homologues of components reported previously in *A. actinomycetemcomitans* (Kachlany et al. 2000) to be involved in the biogenesis of Flp pili. This subfamily of T4b pili has since been reported to contribute to the virulence of several human and animal pathogenic bacteria species (Fuller et al. 2000; Schreiner et al. 2003; Spinola et al. 2003). Why *R. solanacearum* has two distinct *tad* loci is not clear, but phylogenetic analyses have indicated that the *tad* locus has experienced a complex history of duplication, loss, gene shuffling (recombination) and horizontal gene transfer between distant bacterial relatives (Planet et al. 2001, 2003). Nevertheless, the widespread existence of the *tad* locus (Planet et al. 2003; Tomich et al.

2007) suggests that there exists a strong selective pressure for the maintenance of this gene cluster across a diverse spectrum of bacterial species. It is therefore reasonable to assume that the protein products encoded by this gene cluster may be important for some aspect of the life cycle of the organisms that contain these genes. In this study, we have investigated the relevance of the megaplasmid *tad* gene cluster in *R. solanacearum* virulence on potato plants.

Results obtained in this study indicated that mutagenesis of the *tadA2* gene located in the megaplasmid *tad* locus yielded phenotypes that could be rescued if a wild-type copy of the *tadA2* gene was provided *in trans* to the NB336-1085 mutant strain. It is tempting to speculate that the absence of a *tadD* homologue in the chromosomal *tad* locus may prevent the proper assembly of the RcpA secretin of the Flp biogenesis apparatus, thus prohibiting the proper assembly and/or secretion of the Flp pilus. Moreover, *in silico* analyses revealed that the chromosomal *tad* locus has the genetic information to produce three similar Flp-like proteins (RSc0659 lacks an Flp motif). Whether one or more of the putative *flp* genes in this *tad* locus may represent pseudogenes that have become dispensable for Flp pilin biogenesis, as in the case of *flp-2* of *A. actinomycetemcomitans* (Perez et al. 2006), or might still have a function is yet to be determined. Alternatively, it may also be possible that the chromosomal *tad* gene cluster is not expressed under the conditions used in this study. In contrast, although the *tad* locus in the *R. solanacearum* megaplasmid lacks homologues of genes present within the *tad* gene cluster of *A. actinomycetemcomitans* (*e.g.* a second *flp* gene, a second pseudopilin gene and *rcpB* gene), it may still be able to function as an Flp pilus biogenesis and secretion system. This is based on the observation that *P. aeruginosa*, which also lacks homologues of these *tad* genes, is capable of assembling functional Flp pili in their absence (de Bentzmann et al. 2006).

Literature regarding pili in *R. solanacearum* suggests that this bacterium is capable of producing various types of pili. Characterization of *R. solanacearum* strains GMI1000 (van Gijsegem et al. 2000) and AWI (Kang et al. 2002) indicated that they each produce two distinct types of pili, *i.e.* the HrpY pilus encoded by the *hrpY* gene and the T4a pilus encoded by the *pilA* gene. In addition to these reports, Stemmer and Sequeira (1987) reported purified pilin protein from *R. solanacearum* strain K60 of which neither the molecular mass nor the amino acid compositions matched either HrpY or T4a pilins. This result indicates the presence of at least a third distinct type of pilus in *R. solanacearum.* It has been reported that HrpY pili are not involved in adherence (Aldon et al. 2000; van Gijsegem et al. 2000) and the absence of T4a pili was reported to have no quantitative effect on adherence to host or non-host cells (Kang et al. 2002), thus furthermore suggesting the presence of an as yet uncharacterized attachment factor. The results of this study indicate that the *R. solanacearum* megaplasmid *tad* gene cluster may encode a functional system that is responsible for the assembly of Flp pili. In contrast to the wild-type NB336 strain, no Flp-like pili could be seen in TEM preparations of the mutant strain NB336-1085, in which the *tadA2* gene encoding the traffic NTPase was mutated. This is in agreement with previous reports indicating that mutants deficient in TadA are devoid of Flp pili (Kachlany et al. 2000; Bhattarcharjee et al. 2001; Nika et al. 2002; de Bentzmann et al. 2006).

Characterization of the non-polar mutant strain NB336-1085 indicated that it had a mucoid colony morphology on solid medium, grew as well as the wild-type strain in medium and *in planta*, elicited a normal hypersensitivity response, was not affected in its ability to produce EPS galactosamine, and showed wild-type swimming and twitching motility. The wild-type and mutant strains, however, differed from each other with regards to virulence and biofilm

formation. This therefore suggests that a functional *tadA2* gene, and thus Flp pili, plays an important role during disease development.

R. solanacearum enters plants through the roots, penetrates the xylem, systemically colonizes the stem and causes wilt symptoms (Vasse et al. 1995, 2005). During the soil-inhabiting phase, swimming motility mediated by means of flagella (Tans-Kersten et al. 2001), as well as chemotaxis (Yao and Allen 2006) and aerotaxis (Yao and Allen 2007) may be crucial to respond to and move towards gradients of root exudates to start the infection process. Once inside the confined environment of the xylem vessels, twitching motility mediated by T4a pili may be important to the bacterium to spread and colonize other parts of the infected plant and overcome nutrient limitations (Kang et al. 2002). In this study, we have used two different inoculation methods to test the importance of Flp pili during different stages of pathogenesis. In a biologically representative soil soak inoculation assay, the wild-type NB336 strain, but not the mutant NB336-1085 strain, caused wilting of potato plants. These results suggest that the mutant strain did not ingress into potato roots or, alternatively, that the bacteria may have entered plant roots but that they differed from the wild-type strain in their ability to colonize potato stems. The non-wilting phenotype of strain NB336-1085 was observed in both the soil soak and cut petiole inoculation assays, thus indicating a role for Flp pili for successful infection of the host plant beyond the stages of root adhesion and root colonization by the strain.

What might the role of Flp pili be during disease development? The mutant NB336-1085 strain could multiply *in planta* but was unable to cause wilting of the potato plants used in the virulence assays, albeit that localized leaf yellowing and necrosis was observed for cut petiole-inoculated plants. Flp pili might thus take part mainly in the late stages of the disease

development process, possibly by modulating disease severity rather than the infective ability of the bacterium. A study recently reported that posttranslational modification of *Neisseria meningitidis* T4P results in the detachment of bacterial cells from cell aggregates, which, in turn, allows their spread to new colonization sites. In contrast, a mutant strain that was blocked in pilin modification remained attached to the epithelial cells (Chamot-Rooke et al. 2011). In this regard, it is interesting to note that the Flp pilin of both *A. actinomycetemcomitans* and *P. aeruginosa* is glycosylated, and the RcpC protein has been implicated as being required for the synthesis of the modified Flp pili (Tomich et al. 2006; Bernard et al. 2009). The mutant NB336-1085 formed significantly greater biofilms than the wild-type and complemented mutant strains. It is thus tempting to speculate that, whereas loss of the Flp pilin may cause increased aggregation, modification of the Flp pilin in the wild-type may cause reduced aggregation, resulting in the dissemination of individual cells and subsequent colonization of new sites within the infected host plant. This may also have the additional advantage of avoiding nutrient limitation or exhaustion. Whether the above scenario may relate to the situation *in planta* requires further investigation, and microscopy studies are needed to observe biofilm formation in the xylem vessels of host plants infected by the wild-type NB336 and mutant NB336-1085 strains.

The *tad* gene cluster may, however, also play a more direct role in virulence. Most of the proteins encoded by the *tad* locus are evolutionary related to type II and type IV secretion systems (Tomich et al. 2007). In the case of *R. solanacearum*, it is estimated that the bacterium exports large repertoires of pathogenicity effectors through the Type II, in addition to the Type III secretion system (Poueymiro and Genin, 2009). Indeed, mutants unable to secrete Type II secretion-dependent exoproteins lost the ability to cause disease symptoms and to efficiently colonize the plants (Kang et al. 1994). At this stage, we cannot rule out the

possibility that the *R. solanacearum* Tad apparatus also transports virulence factors, as had been suggested previously for the Tad system of the human pathogen *A. actinomycetemcomitans* (Wang and Chen 2005).

Although biofilms are suspected of playing a role in *R. solanacearum*-host interaction (Morris and Monier, 2003), few studies have been undertaken and the factors that affect *R. solanacearum* biofilm formation are still unknown. *R. solanacearum* has been reported to form biofilm-like aggregations on a PVC surface (Kang et al. 2002; Yao and Allen 2007) and on the surface of tomato seedling roots (Kang et al. 2002; Yao and Allen 2006). It has been proposed that once inside the plant, biofilms could help either to protect the pathogen from host defenses and thus contribute to bacterial survival during latent infections and saprophytic life (Stemmer and Sequeira 1987), or it may enable the pathogen to remain anchored to xylem cell walls and filter nutrients from the dilute flow of xylem liquid (Yao and Allen 2007). In this study, both the mutant and the wild-type *R. solanacearum* strains formed biofilms on PVC plastic surfaces at the liquid-air interface. Interestingly, the NB336-1085 mutant strain overproduced biofilms compared to the wild-type NB336 strain. The result was somewhat unexpected, since *tad* mutants of *A. actinomycetemcomitans* and *P. aeruginosa* are impaired in biofilm formation on abiotic surfaces (Kachlany et al. 2000; Perez et al. 2006; Tomich et al. 2006; de Bentzmann et al. 2006). In the case of *R. solanacearum*, the presence of a functional *tadA2* gene thus appears to mask the contribution of other cell surface appendages, such as the T4a pili, to the biofilm formation process. In other words, Flp pili may disturb T4a pili contact with surfaces and therefore in Flp-deficient mutants, T4a pili can promote a stronger attachment to the surface that results in enhanced biofilm formation relative to the wild-type. This suggests that T4a pili, but not Flp pili encoded by the *tad* gene cluster, are important for biofilm formation by *R. solanacearum*. This statement is supported

by the fact that T4a pili mutants of *R. solanacearum* AWI (Kang et al. 2002) and *A. avenae* subsp. *citrulli* (Bahar et al. 2009) are severely impaired in biofilm formation on abiotic surfaces.

In conclusion, the results of this study provide strong support that the *tad* locus in the megaplasmid of *R. solanacearum* is essential for expression of virulence on potato plants. This represents the first report regarding the characterization of a *tad* gene cluster of a phytopathogenic bacterium, and is the first example to indicate that its presence contributes significantly to plant pathogenesis. In addition to *R. solanacearum*, BLAST searches also identified complete *tad* loci in various other gram-negative phytopathogenic bacteria, including *Pseudomonas syringae*, *Agrobacterium tumefaciens*, *Agrobacterium vitis*, *Candidatus liberibacter*, *Pectobacterium carotovorum* subspecies *atrosepticum*, *brasiliensis, carotovorum* and *wasabiae*, as well as in gram-positive phytopathogenic bacteria such as *Clavibacter michiganensis*, *Leifsonia xyli* and *Streptomyces scabiei* (data not shown). The challenge is now to determine precisely what the specific roles of the *R. solanacearum tad* gene products are in the pathogenesis of bacterial wilt. This knowledge will not only enable a better understanding of this important virulence factor, but may also provide significant clues about the functions of the Tad proteins in other phytopathogenic bacteria.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. All *R. solanacearum* strains in this study were derived from wild-type strain NB336 (phylotype II, race 3, biovar 2), obtained from Coen Bezuidenhout Seed Testing Centre (CBSTC, Pretoria,

South Africa). *R. solanacearum* strains were grown in CPG broth (Hendrick and Sequeira 1984) or on TZC agar (Kelman 1954) at 30°C. *Escherichia coli* strains were grown in LB broth (Miller 1992) at 37°C. When appropriate, the *E. coli* growth medium was supplemented with ampicillin (50 µg/ml), tetracycline (10 µg/ml) or gentamycin (7 µg/ml). The *R. solanacearum* growth media were supplemented with tetracycline (10 µg/ml) or gentamycin (15 µg/ml). The growth of *R. solanacearum* wild-type and mutant strains was compared in CPG broth. The ability of the bacterial strains to elicit a hypersensitive reaction was determined by infiltration of tobacco leaves according to the procedures described by Liu et al. (2001). To prepare inoculum, *R. solanacearum* cultures grown overnight in CPG broth were diluted 50-fold in fresh CPG broth and the cultures were incubated until they reached an optical density at 600 nm (OD_{600}) of 0.8. The cells were harvested by centrifugation (4000 × *g*, 25 min, 4°C) and suspended in sterile distilled water (dH_2O) until an OD_{600} of 0.1 (*ca.* 1×10^8 CFU/ml as determined by dilution plating).

Growth *in planta*

The growth of *R. solanacearum* wild-type and mutant strains were compared in 30-day-old potato plants. The potato plants used in these and in virulence assays were grown from seeds of *Solanum tuberosum* BP1, a commercial cultivar obtained from RSA Seeds (Pretoria, South Africa). Stem inoculations were performed, as described previously (Frey et al. 1994; Araud-Razou et al. 1998), by injecting 5 µl of the standardized bacterial suspension (10^8 CFU/ml) into each plant and the inoculated plants were incubated in a greenhouse at 28-30°C. At each time interval post-inoculation the petiole stub was excised and discarded, and 1.5-cm segments of the stem, obtained from above and below the point of inoculation, were excised and surface sterilized by rinsing the stems in sterile dH_2O and then in 70% ethanol. After rinsing again with sterile dH_2O, the stems were homogenized with a Homex 6 homogenizer

(Bioreba, Reinach, Switzerland) in 5 ml of sterile dH_2O. Serial dilutions of the stem homogenates were prepared in sterile dH_2O and plated in triplicate onto TZC to enumerate the viable bacteria.

Bioinformatic analyses

The genome sequence of *R. solanacearum* GMI1000 and its annotation is available at http://sequence.toulouse.inra.fr/R.solanacearum.html. *R. solanacearum* homologues of the *A. actinomycetemcomitans flp-rcp-tad* genes were identified using the BLAST algorithm and the deduced amino acid sequences were subjected to analysis using different software programs, such as BLAST-P, PSORT, SMART tool, SIGNALP, LipoP, TMPRED, ScanProsite, and ClustalW. These programs are available at http://au.expasy.org/tools/.

Recombinant DNA techniques

Molecular cloning techniques used in the construction of recombinant plasmids were carried out using standard procedures (Sambrook and Russell 2001). T4 DNA ligase (Roche Diagnostics, Mannheim, Germany) and restriction enzymes (Fermentas, St. Leon-Rot, Germany) were used according to the manufacturers' protocols. Plasmid DNA was extracted from *E. coli* with a Zyppy Plasmid Miniprep kit, genomic DNA was isolated from *R. solanacearum* strains with a ZR Fungal/Bacterial DNA 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, USA). Plasmid constructions were first established in *E. coli* DH5α and then transferred to *R. solanacearum* strains. Competent cells were prepared and transformed according to published procedures for *E. coli* (Cohen et al. 1972) and *R. solanacearum* (Allen et al. 1991). PCR assays were performed with Biotaq DNA polymerase (Bioline Inc., Randolph, MA, USA) and PCR amplicons were purified with

the QIAquick PCR purification kit (Qiagen, Hilden, Germany). Primers used in this study were designed from the *R. solanacearum* GMI1000 genome sequence and obtained from Integrated DNA Technologies (Coralville, IA, USA). Southern blot hybridization was performed using the DIG-High Prime DNA Labelling 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, USA), followed by resolution on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems), in accordance with the manufacturer's instructions. All plasmid constructs were verified by restriction endonuclease digestion and by nucleotide sequencing.

Construction of *tadA2* mutant NB336-1085

A 2.306-kb DNA fragment containing the putative *tadA2* open reading frame (ORF) flanked by upstream (534 bp) and downstream (422 bp) sequences was PCR amplified from NB336 genomic DNA using primers TadAZ-F (5'-CGTCGAGAATTCCGGGCAGTTGC-3') and TadAB-R (5'-CAACCGCCTGCGAAGCTTCTTCG-3'). The resulting amplicon was digested with both *Eco*RI and *Hind*III (underlined above) and cloned into the identical sites of pUC19 to generate pUC-1085. The recombinant plasmid was digested with *Not*I and *Pst*I, which released a 761-bp fragment from within *tadA2*, and the gentamycin resistance-encoding cassette *aacCI*, excised from pGEM-Gent (Smith 2003) by digestion with *Not*I and *Pst*I, was cloned into the deletion site, creating pUC-1085::Gm^r. The mutant allele was introduced into *R. solanacearum* NB336 and the resulting strains were selected by gentamycin resistance on TZC agar. Allelic replacement of the *tadA2* gene in the *R. solanacearum* megaplasmid was confirmed by PCR and Southern blot hybridization (data not shown). The confirmed *tadA2* mutant was designated NB336-1085.

Complementation of NB336-1085

The 235-bp *lac* promoter of the plasmid pBluescript SKII (+) was PCR amplified with primers LacP-F (5'-CGGTATCTAGATTTTGTTCCCTTTAGTGAG-3') and LacP-R (5'-TAATGCAGCTGGCACGAAAGCTTTCCC-3'). The amplicon was digested with both *Xba*I and *Hind*III (underlined above) and cloned into the identical sites of the broad-host-range cosmid pLAFR6 (Huynh et al. 1989) to generate pLAFR-Lp. A 1.35-kb fragment containing a full-length copy of *tadA2* was PCR amplified from NB336 genomic DNA using primers CpaF2-F (5'-CGACCAGAGCGCGAATTCAGACATGGAATC-3') and CpaF2-R (5'-GGAACGGAACTCTAGATGACACAACCCATCG-3'), digested with both *Eco*RI and *Xba*I (underlined above) and cloned into LAFR-Lp. The complementation plasmid pLAFR-Lp-1085 was introduced into NB336-1085 and complemented strains were selected by tetracycline resistance on TZC agar. The complemented mutant strain was designated NB336-1085comp.

Electron microscopy

Cultures of the *R. solanacearum* strains were prepared for transmission electron microscopy (TEM) according to the methods of Stemmer and Sequeira (1987). The cultures were grown statically at 30°C in sterile glass Petri dishes containing 2 ml of broth (0.1% [w/v] casein hydrolysate, 1% [w/v] peptone, 20 mM glutamate; pH 7.3-7.5). The cultures were transferred twice to fresh broth, using only the pellicle as the inoculum. After 48 h of growth, a 200-mesh Formvar-coated copper grid was floated on the surface of the culture for 5 min and then rinsed with sterile dH_2O to remove debris. The copper grids were negatively stained in 1% uranyl acetate for 30 s, the excess stain was wicked away and the grids were viewed in a JEOL 2100F transmission electron microscope at 80 kV.

Extracellular polysaccharide (EPS) production

EPS was extracted from *R. solanacearum* culture supernatants, as described by Brumbley and Denny (1990), and the concentration of galactosamine was determined using a modified Elson-Morgan reaction and *N*-acetylgalactosamine as standard (Jang et al. 2005).

Motility assays

Motility assays were performed using standardized cultures (OD_{600} = 0.1) of the *R. solanacearum* strains, as described by Liu et al. (2001). Swimming motility was assayed by stab inoculation of motility agar (1% [w/v] tryptone, 0.3% [w/v] agar), followed by incubation of the agar plates for 48 h at 30ºC. Twitching motility was assayed on CPG agar. After incubation overnight at 30°C, colonies were examined for twitching motility by placing a Petri dish, without its lid, on the stage of a Nikon light microscope equipped with a 10× objective (Nikon Optiphot, Tokyo, Japan). The images were acquired with a Nikon DXM 1200 digital camera.

Virulence assays

Virulence tests of *R. solanacearum* strains were conducted on susceptible potato plants, as described by Liu et al. (2001), except that the plants were not watered for 24 h prior to inoculation (Williamson et al. 2002). Potato plants (30-day-old, 15 cm in height) were inoculated by drenching the soil (0.5 kg) in 20-cm pots with 50 ml of the bacterial suspension (10^8 CFU/ml) or by applying 5 µl of the inoculum to the stub of freshly severed leaf petioles. As controls, potato plants were likewise inoculated with sterile dH_2O. The plants were maintained under greenhouse conditions that favor bacterial wilt development, *i.e.* temperature of 28-30ºC, relative humidity of 85-95% and natural day/night cycles (Prior et al.

1996). The plants were rated daily for 30 days using a 0-to-4 disease index, with 0 being healthy (Fock et al. 2000, 2001).

Biofilm formation

Biofilm formation on an abiotic surface was determined using a quantitative plate assay (Yao and Allen 2007) with minor modification. Briefly, a 96-well polyvinyl chloride (PVC) plate (Nunc, Roskilda, Denmark), containing 50 µl of CPG broth per well, was inoculated with 150 µl of standardized cultures (OD_{600} = 0.1) of the *R. solanacearum* strains. The plates were sealed with plastic wrap and incubated without shaking for 48 h at 30ºC. Crystal violet staining and biofilm quantification were performed, as described previously (O'Toole and Kolter 1998), except that the optical density was determined at OD_{600}.

Statistical analyses

Quantitative assays were analyzed by using analysis of variance (ANOVA) at the 95% level, and Tukey's honestly significant difference (HSD) test for mean comparison. All statistical analyses were performed using JMP v.5 software (SAS Institute Inc., Cary, NC, USA).

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LITERATURE CITED

Aldon, D., Brito, B., Boucher, C., and Genin, S. 2000. A bacterial sensor of plant-cell contact controls the transcriptional induction of *Ralstonia solanacearum* pathogenicity genes. EMBO J. 19:2304-2314.

Allen, C. A., Huang, Y., and Sequeira, L. 1991. Cloning of genes affecting polygalacturonase production in *Pseudomonas solanacearum.* Mol. Plant-Microbe Interact. 4:147-154.

Araud-Razou, I., Vasse, J., Montrozier, H., Etchebar, C., and Trigalet, A. 1998. Detection and visualization of the major acidic exopolysaccharide of *Ralstonia solanacearum* and its role in tomato root infection and vascular colonization. Eur. J. Plant Pathol. 104:795-809.

Bahar, O., Goffer, T., and Burdman, S. 2009. Type IV pili are required for virulence, twitching motility, and biofilm formation of *Acidovorax avenae* subsp. *citrulli*. Mol. Plant-Microbe Interact. 22:909-920.

Bernard, C. S., Bordi, C., Termine, E., Filloux, A., and de Bentzmann, S. 2009. Organization and PprB-dependent control of the *Pseudomonas aeruginosa tad* locus, involved in Flp pilus biology. J. Bacteriol. 191:1961-1973.

Bhattacharjee, M. K., Kachlany, S. C., Fine, D. H., and Figurski, D. H. 2001. Nonspecific adherence and fibril biogenesis by *Actinobacillus actinomycetemcomitans*: TadA protein is an ATPase. J. Bacteriol. 183:5927-5936.

Brito, B., Marenda, M., Barberis, P., Boucher, C., and Genin, S. 1999. *prhJ* and *hrpG*, two new components of the plant signal dependent regulatory cascade controlled by PrhA in *Ralstonia solanacearum*. Mol. Microbiol. 31:237-251.

Brumbley, S. M., and Denny, T. P. 1990. Cloning of *phcA* from wild-type *Pseudomonas solanacearum*, a gene that when mutated alters expression of multiple traits that contribute to virulence. J. Bacteriol. 172:5677-5685.

Buttner, D., and Bonas, U. 2002. Getting across - bacterial type III effector proteins on their way to the plant cell. EMBO J. 21:5313-5322.

Chamot-Rooke, J., Mikaty, G., Malosse, C., Soyer, M., Dumont, A., Gault, J., Imhaus, A. F., Martin, P., Trellet, M., Clary, G., Chafey, P., Camoin, L., Nilges, M., Nassif, X., and Duménil, G. 2011. Posttranslational modification of pili upon cell contact triggers *N. meningitidis* dissemination. Science 331:778-782.

Clock, S. A., Planet, P. J., Perez, B. A., and Figurski, D. H. 2008. Outer membrane components of the Tad (tight adherence) secreton of *Aggregatibacter actinomycetemcomitans* J. Bacteriol. 190:980-990.

Cohen, S. N., Chang, A. C. Y., and Hsu, L. 1972. Nonchromosomal antibiotic resistance in bacteria: Genetic transformation of *Escherichia coli* by R-factor DNA. Proc. Natl. Acad. Sci. USA 69:2110-2114.

Craig, L., and Li, J. 2008. Type IV pili: paradoxes in form and function. Curr. Opin. Struct. Biol. 18:267-277.

Cunnac, S., Occhialini, A., Barberis, P., Boucher, C., and Genin, S. 2004. Inventory and functional analysis of the large Hrp regulon in *Ralstonia solanacearum*: identification of

novel effector proteins translocated to plant host cells through the type III secretion system. Mol. Microbiol. 53:115-128.

Danhorn, T., and Fuqua, C. 2007. Biofilm formation by plant-associated bacteria. Annu. Rev. Microbiol. 61:401-422.

de Bentzmann, S., Aurouze, M., Ball, G., and Filloux, A. 2006. FppA, a novel *Pseudomonas aeruginosa* prepilin peptidase involved in assembly of type IVb pili. J. Bacteriol. 188:4851-4860.

Denny, T. P., and Baek, S. R. 1991. Genetic evidence that extracellular polysaccharide is a virulence factor of *Pseudomonas solanacearum*. Mol. Plant-Microbe Interact. 4:198-206.

Denny, T. P., Makini, F. W., and Brumbley, S. M. 1988. Characterization of *Pseudomonas solanacearum* Tn*5* mutants deficient in extracellular polysaccharide. Mol. Plant-Microbe Interact. 1:215-223.

Fernandez, L., Marquez, I., and Guijarro, J. A. 2004. Identification of specific *in vivo*-induced genes in *Yersinia ruckeri* and analysis of ruckerbactin, a catecholate siderophore iron acquisition system. Appl. Environ. Microbiol. 70:5199-5207.

Fock, I., Collonnier, C., Luisetti, J., Purwito, A., Souvannavong, V., Vedel, F., Servaes, A., Ambroise, A., Kodja, H., Ducreux, G., and Sihachakr, D. 2001. Use of *Solanum stenotomum* for introduction of resistance to bacterial wilt in somatic hybrids of potato. Plant Physiol. Biochem. 39:899-908.

Fock, I., Collonnier, C., Purwito, A., Luisetti, J., Souvannavong, V., Vedel, F., Servaes, A., Ambroise, A., Kodja, H., Ducreux, G., and Sihachakr, D. 2000. Resistance to bacterial wilt in somatic hybrids between *Solanum tuberosum* and *Solanum phureja*. Plant Sci. 160:165-176.

Frey, P., Prior, P., Marie, C., Koutoujansky, A., Trigalet-Demery, D., and Trigalet, A. 1994. Hrp-mutants of *Pseudomonas solanacearum* as potential biocontrol agents of tomato bacterial wilt. Appl. Environ. Microbiol. 60:3175-3181.

Fronzes, R., Remaut, H., and Waksman, G. 2008. Architectures and biogenesis of non-flagellar protein appendages in Gram-negative bacteria. EMBO J. 27:2271-2280.

Fuller, T. E., Kennedy, M. J., and Lowery, D. E. 2000. Identification of *Pasteurella multocida* virulence genes in a septicemic mouse model using signature tagged mutagenesis. Microb. Pathog. 29:25-38.

Genin, S., and Boucher, C. A. 2004. Lessons learned from the genome analysis of *Ralstonia solanacearum*. Annu. Rev. Phytopathol. 42:107-134.

Gerlach, R. G., and Hensel, M. 2007. Protein secretion systems and adhesins: the molecular armory of Gram-negative pathogens. Int. J. Med. Microbiol. 297:401-415.

Gonzalez, E. T., and Allen, C. 2003. Characterization of a *Ralstonia solanacearum* operon required for polygalacturonate degradation and uptake of galacturonic acid. Mol. Plant-Microbe Interact. 16:536-544.

Hayward, A. C. 1991. Biology and epidemiology of bacterial wilt caused by *Pseudomonas solanacearum*. Annu. Rev. Phytopathol. 29:65-87.

Henderson, B., Wilson, M., McNab, R., and Lax, A. J. 1999. Cellular microbiology. Bacteria-host interactions in health and disease. John Wiley and Sons, NY, USA.

Hendrick, C., and Sequeira, L. 1984. Lipopolysaccharide-defective mutants of the wilt pathogen *Pseudomonas solanacearum.* Appl. Environ. Microbiol. 48:94-101.

Hikichi, Y., Yoshimochi, T., Tsujimoto, S., Shinohara, R., Nakaho, K., Kanda, A., Kiba, A., and Ohnishi, K. 2007. Global regulation of pathogenicity mechanism of *Ralstonia solanacearum*. Plant Biotechnol. 24:149-154.

Huynh, T. V., Dahlbeck, D., and Staskawicz, B. J. 1989. Bacterial blight of soybean: regulation of a pathogen gene determining host cultivar specificity. Science 245:1374-1377.

Jang, J. H., Hia, H. C., Ike, M., Inoue, C., Fujita, M., and Yoshida, T. 2005. Acid hydrolysis and quantitative determination of total hexosamines of an exopolysaccharide produced by *Citrobacter* spp. Biotechnol. Lett. 27:13-18.

Janse, J. D., van den Beld, H. E., Elphinstone, J., Simpkins, S., Tjou-Tam-Sin, N. A. A., and van Vaerenbergh, J. 2004. Introduction to Europe of *Ralstonia solanacearum* biovar 2, race 3 in pelargonium zonale cuttings. J. Plant Pathol. 86:147-155.

Kachlany, S. C., Planet, P. J., Bhattacharjee, M. K., Kollia, E., DeSalle, R., Fine, D. H., and Figurski, D. H. 2000. Nonspecific adherence of *Actinobacillus actinomycetemcomitans* requires genes widespread in bacteria and archaea. J. Bacteriol. 182:6169-6176.

Kachlany, S. C., Planet, P. J., DeSalle, R., Fine, D. H., Figurski, D. H., and Kaplan, J. 2001. *flp*-1, the first representative of a new pilin gene subfamily, is required for non-specific adherence of *Actinobacillus actinomycetemcomitans*. Mol. Microbiol. 40:542-554.

Kang, Y., Huang, J. Z., Mao, G. Z., He, L. Y., and Schell, M. A. 1994. Dramatically reduced virulence of mutants of *Pseudomonas solanacearum* defective in export of extracellular proteins across the outer membrane. Mol. Plant-Microbe Interact. 7:370-377.

Kang, Y., Liu, H., Genin, S., Schell, M. A., and Denny, P. T. 2002. *Ralstonia solanacearum* requires type IV pili to adhere to multiple surfaces and for natural transformation and virulence. Mol. Microbiol. 2:427-437.

Kram, K. E., Hovel-Miner, G. A., Tomich, M., and Figurski, D. H. 2008. Transcriptional regulation of the *tad* locus in *Aggregatibacter actinomycetemcomitans*: a termination cascade. J. Bacteriol. 190:3859-3868.

Kelman, A. 1954. The relationship of pathogenicity in *Pseudomonas solanacearum* to colony appearance in a tetrazolium medium. Phytopathology 44:693-695.

Liu, H., Kang, Y., Genin, S., Schell, M. A., and Denny, T. P. 2001. Twitching motility of *Ralstonia solanacearum* requires a type IV pilus system. Microbiol. 147:3215-3229.

Liu, H., Zhang, S., Schell, M. A., and Denny, T. P. 2005. Pyramiding unmarked deletions in *Ralstonia solanacearum* shows that secreted proteins in addition to plant cell-wall-degrading enzymes contribute to virulence. Mol. Plant-Microbe Interact. 18:1296-1305.

Mattick, J. S., Whitchurch, C. B., and Alm, R. A. 1996. The molecular genetics of type IV fimbriae in *Pseudomonas aeruginosa*. Gene 179:147-155.

McGarvey, J. A., Denny, T. P., and Schell, M. A. 1999. Spatial-temporal and quantitative analysis of growth and EPS production by *Ralstonia solanacearum* in resistant and susceptible tomato cultivars. Phytopathology 89:1233-1239.

Miller, J. H. 1992. A short course in bacterial genetics: A laboratory manual and handbook for *Escherichia coli* and related bacteria. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.

Morris, C. E., and Monier, J. M. 2003. The ecological significance of biofilm formation by plant-associated bacteria. Annu. Rev. Phytopathol. 41:429-453.

Nika, J. R., Latimer, J. L., Ward, C. K., Blick, R. J., Wagner, N. J., Cope, L. D., Mahairas, G. G., Munson, R. S., and Hansen, E. J. 2002. *Haemophilus ducreyi* requires the *flp* gene cluster for microcolony formation *in vitro*. Infect. Immun. 70:2965-2975.

O'Toole, G. A., and Kolter, R. 1998. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. Mol. Microbiol. 30:295-304.

Pelicic, V. 2008. Type IV pili: e pluribus unum? Mol. Microbiol. 68:827-837.

Perez, B. A., Planet, P. J., Kachlany, S. C., Tomich, M., Fine, D. H., and Figurski, D. H. 2006. Genetic analysis of the requirement for *flp-2*, *tadV*, and *rcpB* in *Actinobacillus actinomycetemcomitans* biofilm formation. J. Bacteriol. 188:6361-6375.

Planet, P. J., Kachlany, S. C., DeSalle, R., and Figurski, D. H. 2001. Phylogeny of genes for secretion NTPases: identification of the widespread *tadA* subfamily and development of a diagnostic key for gene classification. Proc. Natl. Acad. Sci. USA 98:2503-2508.

Planet, P. J., Kachlany, S. C., Fine, D. H., DeSalle, R., and Figurski, D. H. 2003. The widespread colonization island of *Actinobacillus actinomycetemcomitans*. Nature Genet. 34:193-198.

Possot, O., and Pugsley, A. P. 1994. Molecular characterization of PulE, a protein required for pullulanase secretion. Mol. Microbiol. 12:287-299.

Poueymiro, M., and Genin, S. 2009. Secreted proteins from *Ralstonia solanacearum*: a hundred tricks to kill a plant. Curr. Opin. Microbiol. 12:44-52.

Prior, P., Bart, S., Leclercq, S., Darrasse, A., and Anais, G. 1996. Resistance to bacterial wilt in tomato as discerned by spread of *Pseudomonas* (*Burkholderia*) *solanacearum* in the stem tissues. Plant Pathol. 45:720-726.

Salanoubat, M., Genin, S., Artiguenave, F., Gouzy, J., Mangenot, S., Arlat, M., Billault, A., Brottier, P., Camus, J., Cattolico, L., Chandler, M., Choisne, N., Claudel-Renard, C., Cunnac, S., Demange, N., Gaspin, C., Lavie, M., Moisan, A., Robert, C., Saurin, W., Schiex, T., Siguier, P., Thebault, P., Whalen, M., Wincker, P., Levy, M., Weissenbach, J., and Boucher, C. A. 2002. Genome sequence of the plant pathogen *Ralstonia solanacearum*. Nature 415:497-502.

Sambrook, J., and Russel, D. W. 2001. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.

Schell, M. A. 2000. Control of virulence and pathogenicity genes of *Ralstonia solanacearum* by an elaborate sensory network. Annu. Rev. Phytopathol. 38:263-292.

Schreiner, H. C., Sinatra, K., Kaplan, J. B., Furgang, D., Kachlany, S. C., Planet, P. J., Perez, B. A., Figurski, D. H., and Fine D. H. 2003. Tight-adherence genes *of Actinobacillus actinomycetemcomitans* are required for virulence in a rat model. Proc. Natl. Acad. Sci. USA 100:7295-7300.

Smith, J. J. 2003. Towards the development of a biofilm-specific expression system: effects of different promoters, gene dosage and growth phases on extracellular expression of *Pseudomonas aeruginosa* DSM1707 alkaline protease. M.Sc Thesis, Department of Microbiology and Plant Pathology, University of Pretoria, South Africa.

Spinola, S. M., Fortney, K. R., Katz, B. P., Latimer, J. L., Mock, J. R., Vakevainen, M., and Hansen, E. J. 2003. *Haemophilus ducreyi* requires an intact *flp* gene cluster for virulence in humans. Infect. Immun. 71:7178-7182.

Stemmer, W. P. C., and Sequeira, L. 1987. Fimbriae of phytopathogenic and symbiotic bacteria. Phytopathology 77:1633-1639.

Swanson, J. K., Yao, J., Tans-Kersten, J. K., and Allen, C. 2005. Behavior of *Ralstonia solanacearum* race 3 biovar 2 during latent and active infection of geranium. Phytopathology 95:136-143.

Tans-Kersten, J., Huang, H. Y., and Allen, C. 2001. *Ralstonia solanacearum* needs motility for invasive virulence on tomato. J. Bacteriol. 183:3597-3605.

Tomich, M., Fine, D. H., and Figurski, D. H. 2006. The TadV protein of *Actinobacillus actinomycetemcomitans* is a novel aspartic acid prepilin peptidase required for maturation of the Flp1 pilin and TadE and TadF pseudopilins. J. Bacteriol. 188:6899-6914.

Tomich, M., Planet, P. J., and Figurski, D. H. 2007. The *tad* locus: postcards from the widespread colonization island. Nature Rev. Microbiol. 5:363-375.

van Gijsegem, F., Vasse, J., Camus, J. C., Marenda, M., and Boucher, C. 2000. *Ralstonia solanacearum* produces *hrp*-dependent pili that are required for PopA secretion but not for attachment of bacteria to plant cells. Mol. Microbiol. 36:249-260.

Vasse, J., Danoun, S., and Trigalet, A. 2005. Microscopic studies of root infection in resistant tomato cultivar Hawaii7996. Pages 285-291. In: Allen, C., Prior, P. and Hayward, A. C. (Eds.), Bacterial wilt disease and the *Ralstonia solanacearum* species complex. American Phytopathological Society, St. Paul, MN, USA.

Vasse, J., Frey, P., and Trigalet, A. 1995. Microscopic studies of intercellular infection and protoxylem invasion of tomato roots by *Pseudomonas solanacearum.* Mol. Plant-Microbe Interact. 8:241-251.

Wall, D., and Kaiser, D. 1999. Type IV pili and cell motility. Mol. Microbiol. 32:1-10.

Wang, Y., and Chen, C. 2005. Mutation analysis of the *flp* operon in *Actinobacillus actinomycetemcomitans*. Gene 351:61-71.

Whitchurch, C. B., Hobbs, M., Livingston, S. P., Krishnapillai, V., and Mattick, J. S. 1991. Characterization of a *Pseudomonas aeruginosa* twitching motility gene and evidence for a specialized protein export system widespread in eubacteria. Gene 101:33-44.

Williamson, L., Hudelson, B. D., and Allen, C. 2002. *Ralstonia solanacearum* strains isolated from geranium belong to Race 3 and are pathogenic on potato. Plant Dis. 86:987-991.

Yao, J., and Allen, C. 2006. Chemotaxis is required for virulence and competitive fitness in the bacterial wilt pathogen *Ralstonia solanacearum*. J. Bacteriol. 188:3697-3708.

Yao, J., and Allen, C. 2007. The plant pathogen *Ralstonia solanacearum* needs aerotaxis for normal biofilm formation and interactions with its tomato host. J. Bacteriol. 189:6415-6424.

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics[a]	Reference or source
Strains		
E. coli		
DH5α	F⁻ *recA1 endA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1* Δ (*lacZYA-argF*)U169 λ⁻ [Φ80d*lacZ*ΔM15]	Invitrogen
R. solanacearum		
NB336	Wild-type, virulent potato isolate (race 3, biovar 2, phylotype II); Swm^+, Twt^+, HR^+, EPS^+	CBSTC, Pretoria
NB336-1085	NB336 insertional mutant defective in *tadA2*; Gm^r, Swm^+, Twt^+, HR^+, EPS^+	This study
NB336-1085comp	NB336-1085 complemented with pLAFR-Lp-1085; Gm^r, Tc^r	This study
Plasmids		
pUC19	Cloning vector; ColE1, Amp^r, LacZα peptide	Stratagene
pUC19-1085	pUC19 containing 2.306-kb amplicon harboring full-length *tadA2* and flanking up- and downstream regions, cloned into the *Eco*RI and *Hind*III sites of pUC19	This study
pGEM-Gent	pGEM-T Easy vector containing gentamycin resistance (Gm^r) cassette	Smith (2003)
pUC19-1085::Gm^r	pUC19-1085 with a Gm^r cassette inserted at the *Not*I and *Pst*I sites of *tadA2*	This study
pBluescript SKII (+)	Cloning vector; ColE1, Amp^r, LacZα peptide	Stratagene
pLAFR6	Inc P, RK2-derived cosmid vector; Tc^r	Huynh et al. (1989)
pLAFR-Lp	*lac* promoter (235 bp) amplicon from pBluescript SKII (+) cloned into the *Hind*III and *Xba*I sites of pLAFR6	This study
pLAFR-Lp-1085	pLAFR-Lp with the 1.35-kb *tadA2* gene fragment cloned into the *Xba*I and *Eco*RI sites downstream of the *lac* promoter	This study

[a] Swm, swimming motility; Twt, twitching motility; HR, hypersensitive response on tobacco; EPS, exopolysaccharide; Amp^r, Gm^r, Tc^r, resistance to ampicillin, gentamycin and tetracycline, respectively.

Table 2 Characteristics of the *R. solanacearum tad* loci

Chromosome *tad* locus			Megaplasmid *tad* locus			
Gene name	**RSc number[a]**	**Protein size (aa)**	**Gene name**	**RSp number[a]**	**Protein size (aa)**	**Predicted function**
flp-1	0661	53	*flp*	1092	58	Pilin
flp-2	0660	53				Pilin
flp-3	0659	45				Pilin
flp-4	0658	53				Pilin
tadV1	0657	172	*tadV2*	1091	168	Prepilin peptidase
			tadF	1090	163	Pseudopilin
rcpC1	0656	288	*rcpC2*	1089	208	
				1088	105	
rcpA1	0655	634	*rcpA2*	1087	454	Secretin
	0654	102				
tadZ1	0653	397	*tadZ2*	1086	439	
tadA1	0652	453	*tadA2*	1085	450	ATPase
tadB1	0651	325	*tadB2*	1084	308	PilC-like
tadC1	0650	315	*tadC2*	1083	326	PilC-like
			tadD	1082	305	Pilotin
				1081	109	
tadE	0649	144				Pseudopilin
tadG1	0648	347	*tadG2*	1080	540	
				1079	467	

[a] The RSp and RSc numbers correspond to the genome annotation of *R. solanacearum* strain GMI1000 (http://sequence.toulouse.inra.fr/R.solanacearum.html).

FIGURE CAPTIONS

Fig. 1. The *tad* gene clusters of *R. solanacearum*. (A) Genetic organization of megaplasmid and chromosomal *tad* gene clusters in *R. solanacearum* GMI1000 compared to the homologous gene cluster of *A. actinomycetemcomitans* (Kram et al. 2008). ORFs with similar predicted protein products are indicated in the same color. The transcriptional orientation and approximate size of the different ORFs are indicated by the direction and the length of the arrows. ORFs that are unique to the *R. solanacearum* megaplasmid and chromosomal *tad* loci are indicated by dashed outlines. (B) Alignment of the putative Flp prepilin proteins of *R. solanacearum* and Flp-1 of *A. actinomycetemcomitans*. The Flp motif is underlined and the conserved glycine (G), glutamate (E) and tyrosine (Y) residues in the Flp motif, as well as the phenylalanine (F) residue frequently found within the C-terminal region of Flp pili are indicated by asterisks. The putative Flp prepilin proteins encoded by the megaplasmid and chromosomal *tad* loci of *R. solanacearum* are indicated by a circle and squares, respectively. For all sequences included in the analysis, the GenBank accession numbers are indicated in brackets.

Fig. 2. Transmission electron microscopy of *R. solanacearum* strains, following growth for 48 h in broth supplemented with glutamate (20 mM). Micrographs are shown of negatively stained wild-type NB366, mutant NB336-1085 and complemented mutant NB336-1085comp bacterial cells (A) and loose fimbriae detected in the growth medium of the respective *R. solanacearum* strains (B). Flagella (dashed arrows) and abundant weakly contrasted pili (solid arrows) could be observed in negative preparations of all these *R. solanacearum* strains, whereas the mutant NB336-1085 strain lacked the well-contrasted bundled pili that we referred to as Flp pili (arrowheads). (C) An enlarged view of a Flp fibril produced by the

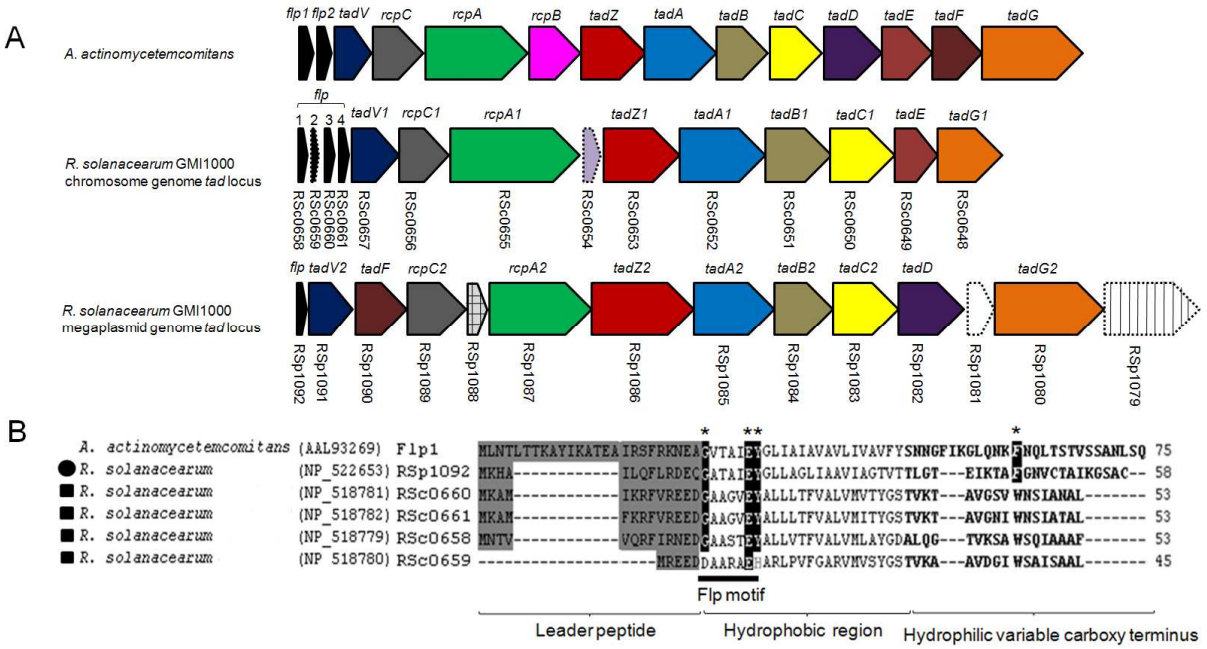
wild-type NB336 strain (boxed area in B), which is comprised of a parallel array of six thin tightly packed individual pili.

Fig. 3. *In planta* growth of the *R. solanacearum* wild-type NB336 and mutant NB336-1085 strains. The stems of potato plants were inoculated with 5×10^5 bacteria and the plants were incubated in a greenhouse at 28-30°C. At 10-h time intervals post-inoculation, 1.5-cm stem segments above (A) and below (B) the point of inoculation were excised, surface sterilized, homogenized and the number of bacterial CFU recovered was determined by dilution plating onto TZC agar medium. The results are the mean of three independent experiments and the error bars represent the standard error of the mean. The growth of the respective strains were not significantly different ($P = 0.05$) at any of the time points according to Tukey's HSD test.

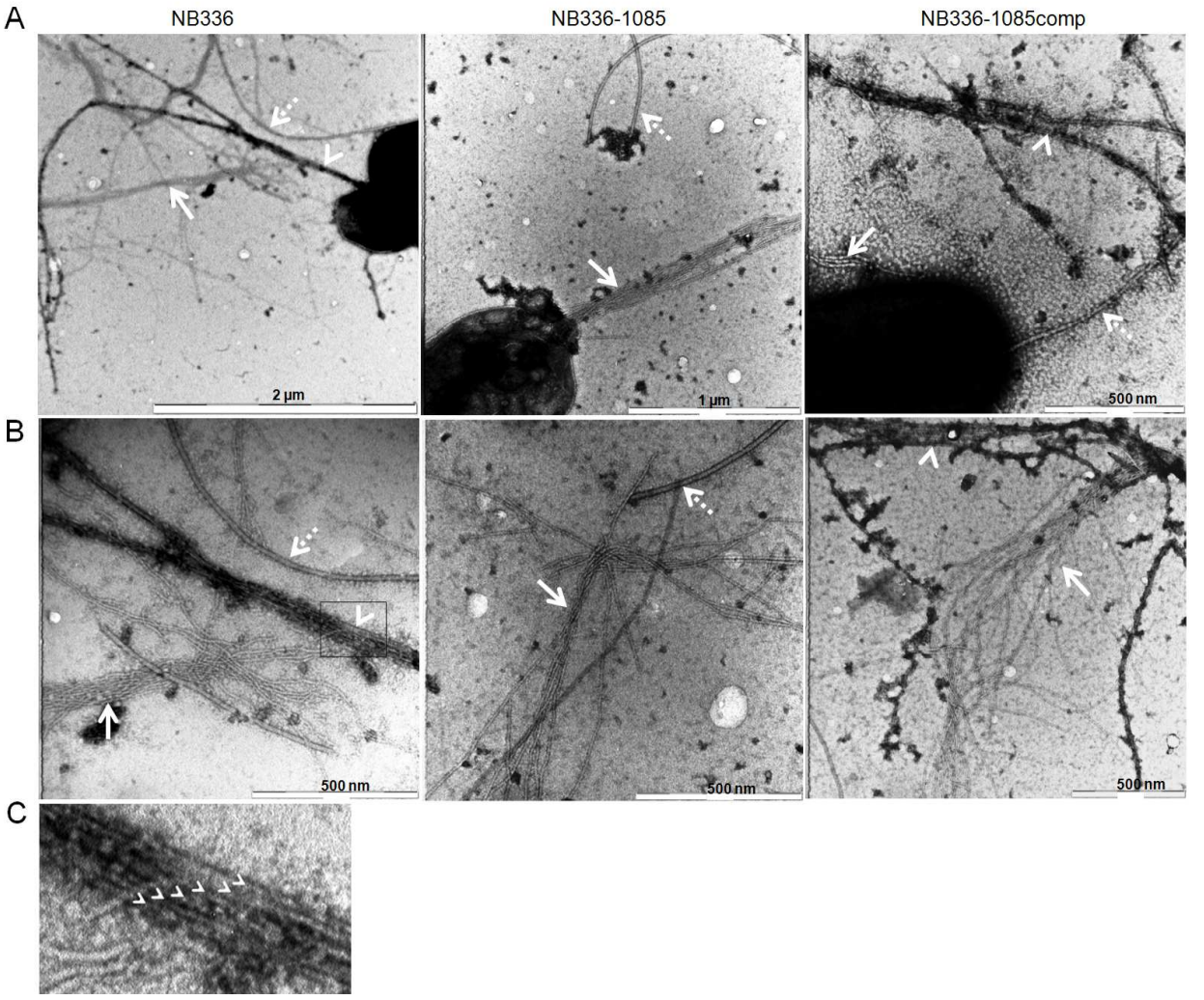
Fig. 4. Disease progress of the *R. solanacearum* strains on potato plants by different inoculation methods. Thirty-day-old potato plants were inoculated either by soaking the soil to a final bacterial population of about 1×10^8 CFU/g of soil (A) or by applying 5×10^5 bacteria directly to the cut surface of a leaf petiole (B). In these assays, plants inoculated with sterile dH_2O were included as a negative control. Plants were rated daily on a disease index scale from 0 to 4. Each point represents the mean disease index of three individual experiments, each containing five plants per treatment. In (A), the virulence of the wild-type and the complemented mutant strains was significantly different from that of the mutant NB336-1085 strain, and the virulence of the complemented strain was significantly different from the wild-type strain according to Tukey's HSD test performed at each days 8-23 ($P = 0.05$). In (B), the virulence of the wild-type and complemented mutant strains was not significantly different, although both differed significantly from that of the mutant NB336-1085 strain ($P = 0.05$). (C) Disease symptoms of potato plants inoculated with the *R.*

solanacearum strains by cut-petiole inoculation. Pictures were taken at 10 days post-inoculation of potato plants used in the virulence assay presented in Fig. 4B. Potato plants inoculated with the wild-type NB336 strain and the complemented mutant strain NB336-1085comp showed green and yellow wilting, respectively, whereas potato plants inoculated with the mutant NB336-1085 strain exhibited restricted leaf yellowing but could be maintained until maturity without dying. Potato plants inoculated with sterile dH_2O was used as a negative control.

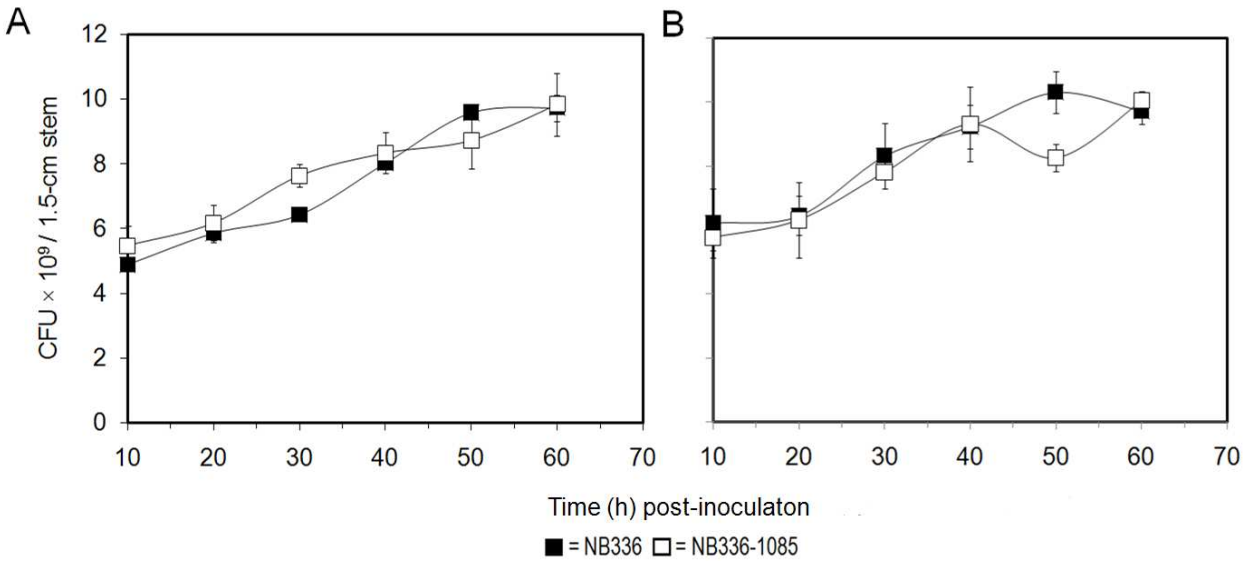
Fig. 5. Biofilm formation by *R. solanacearum* strains. Biofilm formation was quantified by measuring the OD_{600} of crystal violet-stained PVC microtiter wells at 48 h post-incubation. The results are the mean of three independent experiments with five replicates each and the error bars represent the standard error of the mean. Different letters indicate significant differences ($P = 0.05$) among the strains according to Tukey's HSD test.



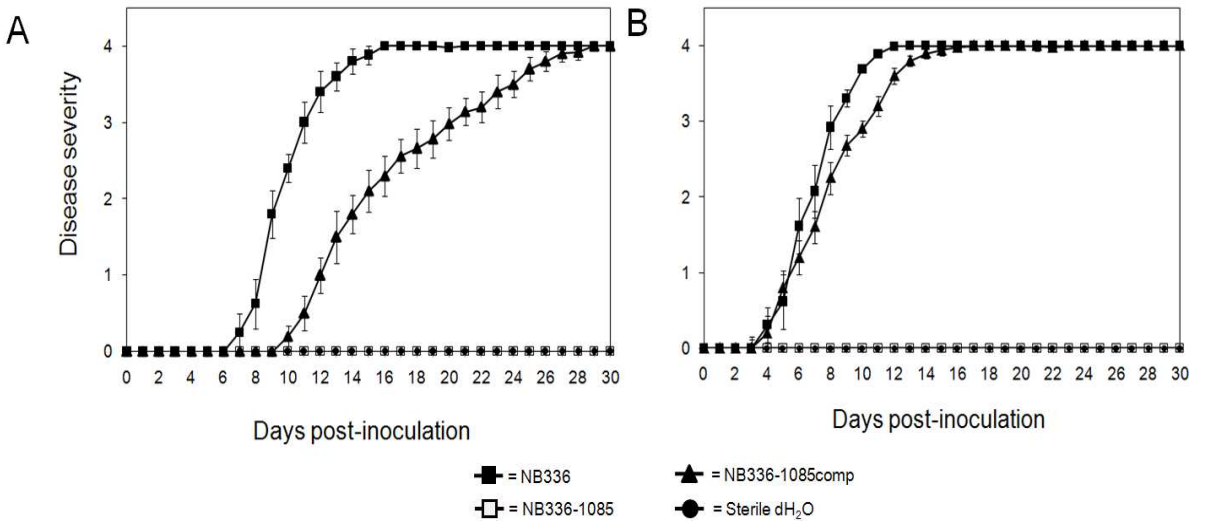
Charles K. Wairuri Fig. 1 *MPMI*

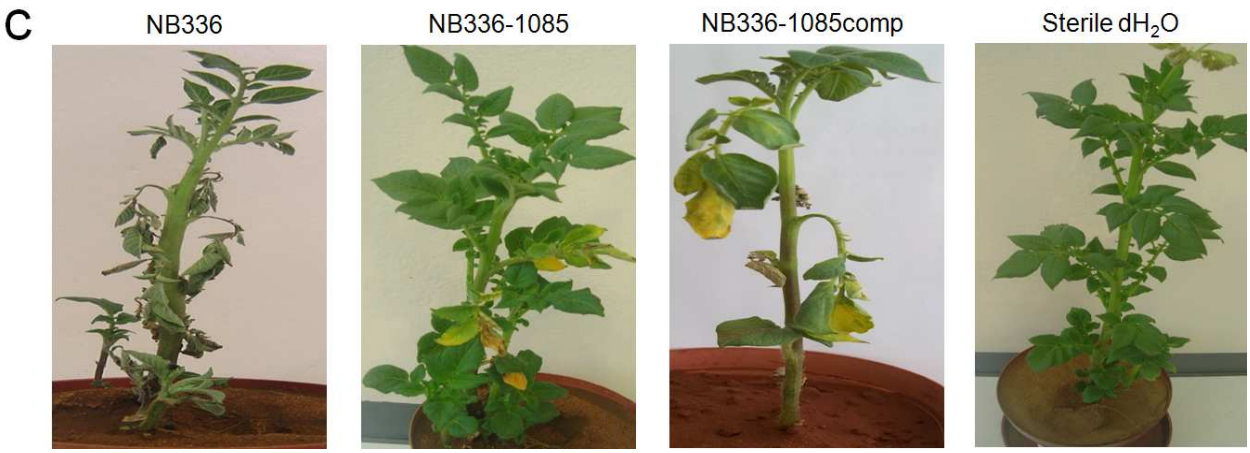


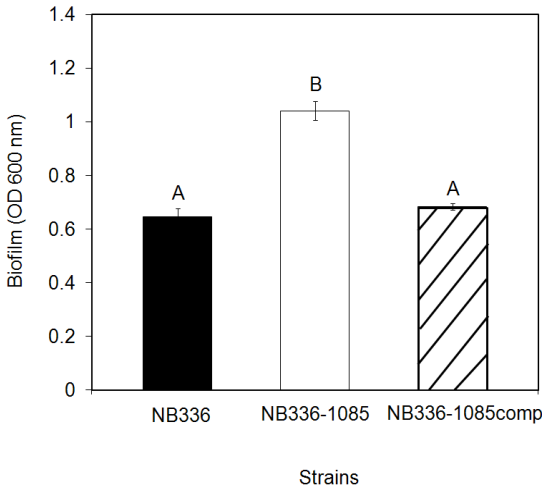
Charles K. Wairuri Fig. 2 *MPMI*

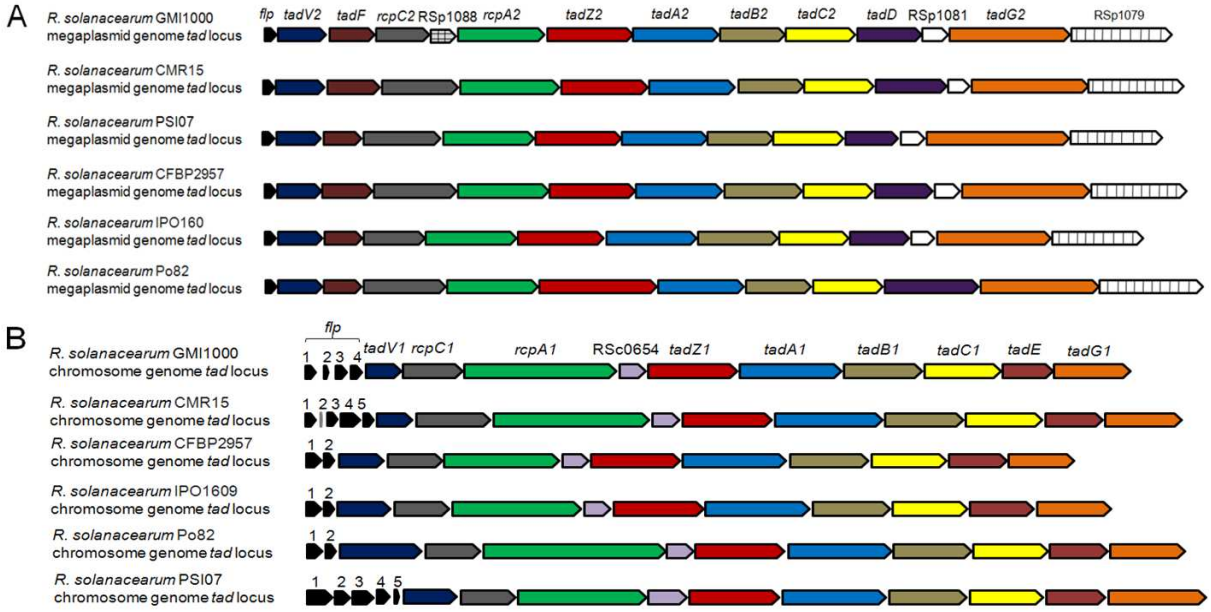


Charles K. Wairuri Fig. 3 *MPMI*

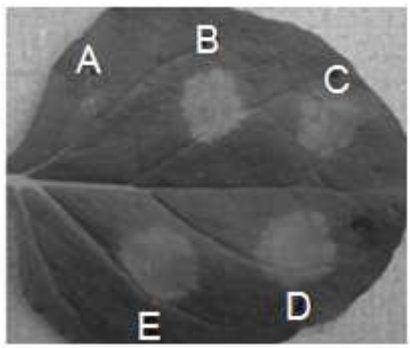

A
Disease severity
0
1
2
3
4
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
Days post-inoculation
= NB336
= NB336-1085
B
0
1
2
3
4
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
Days post-inoculation
= NB336-1085comp
= Sterile dH2O


C
NB336
NB336-1085
NB336-1085comp
Sterile dH2O

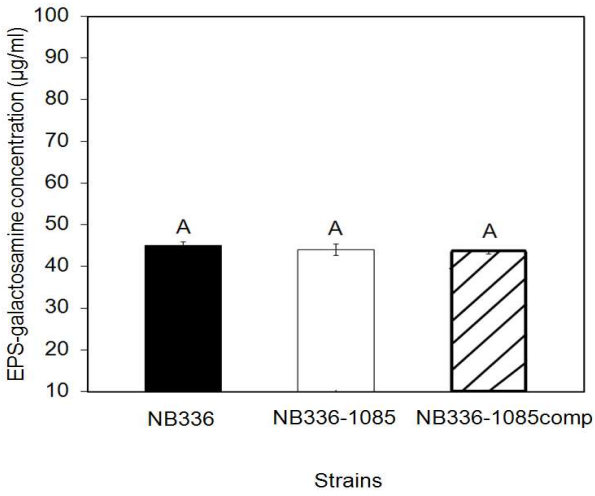

1.4
1.2
1
0.8
0.6
0.4
0.2
0
Biofilm (OD 600 nm)
A
B
A
NB336
NB336-1085
NB336-1085comp
Strains



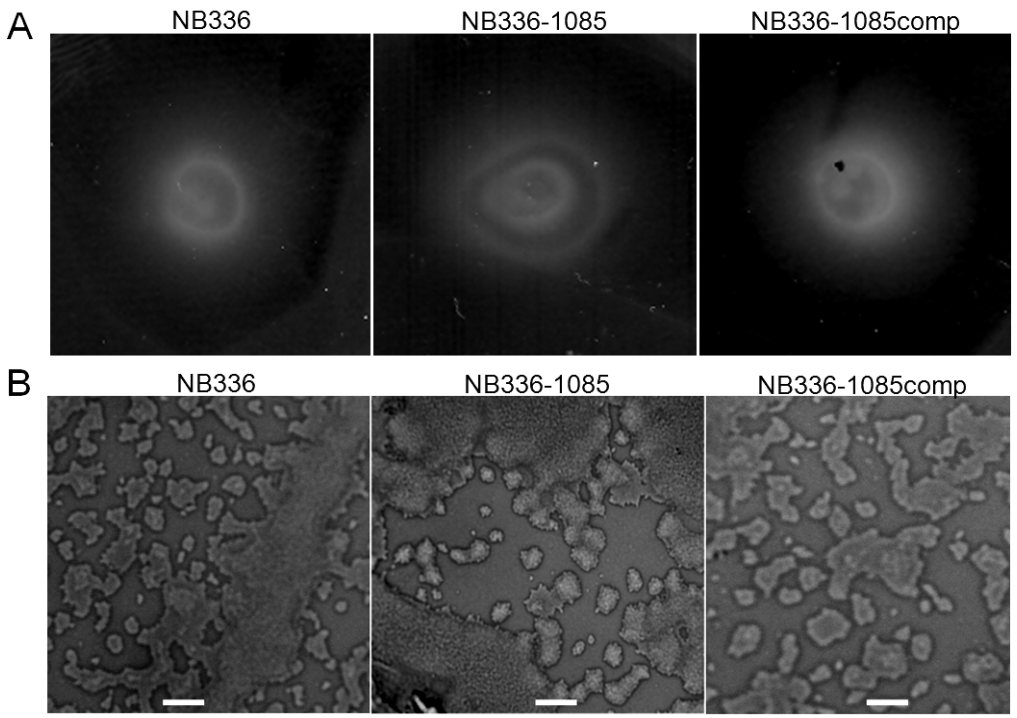
Supplementary Fig. 1. Arrangement of the *tad* genes in *R. solanacearum* GMI1000 and other *R. solanacearum* strains. The genetic organization of the *tad* gene clusters in the megaplasmid (A) and chromosome (B) genomes are shown, with the top line showing the *flp-rcp-tad* genes of *R. solanacearum* GMI1000. ORFs with similar predicted protein products (as determined by BLAST analyses) are indicated in the same color. The sequences are from the following sources, with the GenBank accession no. indicated in brackets: *R. solanacearum* GMI1000 (NC_003296, NC_003295), *R. solanacearum* CMR15 (FP885896, FP885895), *R. solanacearum* CFBP2957 (NC_014309, NC_014307), *R. solanacearum* IPO1609 (NW_002196569), *R. solanacearum* Po82 (CP002819) and *R. solanacearum* PSI07 (NC_014310, NC_014311).



Supplementary Fig. 2. Hypersensitivity reaction (HR)-dependent lesion formation in a tobacco plant inoculated with various bacterial strains. The tobacco plant leaf was infiltrated with standardized suspensions of the *R. solanacearum* wild-type NB336 (C), mutant NB336-1085 (D) and complemented mutant NB336-1085comp (E) strains. As controls, the leaf was also infiltrated with sterile dH_2O (A) and *Xanthomonas campestris* (B). The photographs show lesion after seven days of incubation.



Supplementary Fig. 3. EPS galactosamine production by *R. solanacearum* strains. The bacterial strains were cultured for 96 h at 30°C prior to determining the concentration of EPS galactosamine. The results are the mean of three independent experiments and the error bars represent the standard error of the mean. Differences in EPS galactosamine production were not significant at $P = 0.05$ according to Tukey's HSD test.



Supplementary Fig. 4. Motility assays of *R. solanacearum* strains. (A) For flagellar-mediated swimming motility, the bacterial strains were stab inoculated into motility agar and the zone of colonization was compared after incubation for 48 h at 30°C. (B) For twitching motility, the bacterial strains were cultured on CPG agar and examined 24 post-inoculation under a light microscope. Bar, 10 μm.