Characterization of a novel pilus system in *Ralstonia solanacearum* and its contribution to virulence on potato

by

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DECLARATION

I declare that the dissertation, which I hereby submit for the degree M.Sc (Microbiology) at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution and the work from other sources has been duly acknowledged.

SIGNATURE: ……

Charles Kamau Wairuri

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To God for always protecting us.

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SUMMARY

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Ralstonia solanacearum, a widely distributed soil-borne pathogen belonging to the β-subdivision of Proteobacteria, causes a lethal wilting disease of more than 450 plants species, including economically important crops such as tomato and potato. Although bacterial wilt pathogenesis is incompletely understood, genetic and molecular studies have implicated type IVa pili-mediated twitching motility, amongst other, as a factor contributing to disease development. Research performed over the last decade has led to the identification of the Tad (tight adherence) macromolecular transport system that is present in many bacterial and archaeal species. The *tad* genes encode the machinery required for the biogenesis and secretion of a novel type IVb pilin protein, designated Flp, which is required for colonization and virulence in several human pathogenic bacteria. Therefore, the aims of this investigation were essentially to mine the genome of *R. solanacearum* in order to identify homologues of the *tad* genes and to determine the importance of the Tad system in *R. solanacearum* virulence on potato.

During the course of this study, two distinct *tad* gene clusters were identified in the genome of *R. solanacearum*. These *tad* loci are present in the megaplasmid and chromosome, respectively, and both gene clusters encode predicted protein products with homology to those encoded by the *tad* loci in human pathogenic bacteria such as *Aggregatibacter actinomycetemcomitans* and *Pseudomonas aeruginosa*. Towards determining the importance of the *tad* loci in *R. solanacearum* virulence, a mutation was introduced into the *cpaF2* (RSP1085) gene of the megaplasmid *tad* locus, which encodes a putative NTPase and is highly conserved in different bacterial Tad systems. Comparative analysis of the *R. solanacearum* wild-type NB336 and mutant NB336Δ*cpaF2* strains indicated that the mutant strain was not growth impaired *in vitro* or *in planta*, produced near wild-type levels of EPS, and exhibited swimming and twitching motility that was comparable to that of the wild-type strain. However, the mutant NB336Δ*cpaF2* strain, in contrast to the wild-type NB366 strain, was significantly impaired in its ability to adhere to and colonize potato roots. Moreover, in a biologically representative soil soak inoculation virulence assay, the mutant NB336Δ*cpaF2* strain was not able to cause disease on potato plants, and similar results were obtained when the mutant strain was inoculated directly onto cut potato leaf petioles. Notably, both the adherence and virulence phenotypes were restored when a wild-type copy of the *cpaF2* gene was provided *in trans*. These results suggest that the Tad system located on the megaplasmid, makes an important contribution to *R. solanacearum* virulence on potato. It is likely that this contribution is of importance during the early stages in host plant colonization.

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LIST OF ABBREVIATIONS

%	percentage
°C	degrees Celsius
µg	microgram
µl	microlitre
µm	micrometre
µM	micromolar
× *g*	centrifugal force
A	absorbance
Å	angstrom
Bfp	bundle forming pili
bp	base pair
BLAST	Basic Local Alignment Search Tool
C	carboxy
ca.	approximately
CFU	colony forming units
cm	centimetre
CPG	casein hydrolysate-peptone-glucose
dH_2O	distilled water
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside-5'-triphosphate
DTT	dithiothreitol
EDTA	ethylenediaminetetra-acetic acid
e.g.	for example
EPS	exopolymeric substances
EPS I	exopolysaccharide I
Fig.	figure
g	gram
h	hour
HCl	hydrochloric acid
HR	hypersensitive response
IPTG	isopropyl-β-D-thiogalactoside
kb	kilobase pairs

kDa	kilodalton
kg	kilogram
kV	kilovolt
LB broth	Luria-Bertani broth
M	molar
Mb	megabase pairs
mg	milligram
min	minute
ml	millilitre
mm	millimetre
mM	millimolar
N	amino
NaCl	sodium chloride
NaOAC	sodium acetate
NaOH	sodium hydroxide
ng	nanogram
NJ	Neighbor Joining
nm	nanometre
no.	number
NTPase	nucleoside triphosphate hydrolase
OD	optical density
ORF	open reading frame
PCR	polymerase chain reaction
pmol	picomole
PVC	polyvinyl chloride
RSC	*Ralstonia solanacearum* chromosome
RSP	*Ralstonia solanacearum* megaplsmid
s	second
SDS	sodium dodecyl sulphate
SEM	scanning electron microscopy
TEM	transmission electron microscopy
T4P	type IV pilus
T4ap	type IVa pili
T4bp	type IVb pili
T2S	type II secretion
T3S	type III secretion
T4S	type IV secretion

Tcp	toxin-coregulated pili
TPR	tetratricopeptide repeat
Tris-HCl	Tris (hydroxymethyl) aminomethane hydrochloride
TZC	2, 3, 5-triphenyltetrazolium chloride
U	unit
UV	ultraviolet
V	volts
v.	version
v/v	volume per volume
VWA	von Willebrand factor A
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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CHAPTER ONE

LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

Ralstonia solanacearum is a rod-shaped, Gram-negative β-proteobacterium. It is a water- and soil-borne phytopathogen, and a causal agent of bacterial wilt disease (Smith, 1896). The bacterium was previously called *Pseudomonas solanacearum* and formerly *Burkholderia solanacearum* (Yabuuchi *et al.*, 1995). Based on additional phylogenetic and polyphasic phenotype analyses, it was suggested that *Pseudomonas solanacearum* and other *Pseudomonas* species, such as *Pseudomonas eutropha* and *Pseudomonas picketti*, be placed into a new genus, called *Ralstonia* (Gillis *et al.*, 1995; Yabuuchi *et al.*, 1995). Traditionally, *R. solanacearum* has been classified into races (Buddenhagen *et al.*, 1962; Buddenhagen and Kelman, 1964) and biovars (Hayward, 1964), but, more recently, it was genetically grouped into phylotypes (Fegan and Prior, 2005).

Due to a lack of detailed understanding of its pathogenicity and survival strategies, *R. solanacearum* continues to be a devastating phytopathogen (van Elsas *et al.*, 2000; Vasse *et al.*, 2005). In the case of *R. solanacearum,* successful pathogenesis requires adherence to plant tissues and subsequent colonization of xylem vessels, which contributes to vascular dysfunction (Kao *et al.*, 1992; Schell, 2000). Adherence of the bacterium is mediated by flagella and type IVa pili (T4ap) (Liu *et al.*, 2001; Tans-Kersten *et al.*, 2001; Kang *et al.*, 2002). In 2000, a novel Type IVb pilus (T4bp) biogenesis and secretion system was described in *Aggregatibacter actinomycetemcomitans* (Kachlany *et al.*, 2000). This pilus biogenesis and secretion system has since been reported in many other bacteria (Planet *et al.*, 2003; Tomich *et al.*, 2007), and was also shown to be associated with adherence in several bacterial species (Nika *et al.*, 2002; de Bentzmann *et al.*, 2006; Perez *et al.*, 2006).

Pili are filamentous multimeric macromolecules and are synthesized through the ordered polymerization of pilin subunits. In general, the bacterial pilus is composed of a repeating polypeptide packed into a helical assembly of which the tip may or may not display a protein adhesin (Pugsley, 1993; Marceau *et al.*, 1998; Henderson *et al.*, 1999). Their biogenesis involves many genes, including those that encode the major subunit, proteins required for biogenesis and assembly, and regulatory proteins (Craig and Li, 2008). As the involvement of pili in the promotion of *R. solanacearum* pathogenesis is closely aligned with the aims of this investigation, various aspects regarding pili and their assembly, as well as their contribution to disease development will be discussed in greater detail in this review of the literature.

1.2 CLASSIFICATION OF *R. solanacearum*

The *R. solanacearum* species complex includes a heterogeneous group of isolates with important pathogenic and genotypic differences (Fegan *et al.*, 1998b; Fegan and Prior, 2005). The initial classification of *R. solanacearum* was according to their pathogenicity on different hosts (Buddenhagen *et al.*, 1962; Buddenhagen and Kelman, 1964). Based on host range, *R. solanacearum* can be differentiated into five races. Strains belonging to race 1 have a very broad host range and are commonly endemic to America. Race 2 strains are pathogenic only to Musaceae (triploid banana and other *Musa* species), and are present in Central America and Southeast Asia. Race 3 strains are widespread all over the world and pathogenic to solanaceous plants, such as tomato and potato, and non-solanaceous plants, including ornamentals, such as geranium (Williamson *et al.*, 2002; Janse *et al.*, 2004; Swanson *et al.*, 2005). Race 4 strains are pathogenic to ginger in Asia, whereas race 5 strains are pathogenic to mulberry in China. However, the race classification system of *R. solanacearum* is poorly defined due to the overlap between host ranges and is thus taxonomically not useful (Hayward, 1964; Gabriel *et al.*, 2006). Nevertheless, it is apparent that different races exhibit variation not only in host range, but also in their geographic distribution (Fegan and Prior, 2005).

Due to the shortcomings associated with the above classification system, *R. solanacearum* has been reclassified into biovars on the basis of their metabolic properties. Based on the oxidation of six different carbon sources, namely maltose, lactose, cellobiose, mannitol, sorbitol and dulcitol, *R. solanacearum* strains were separated into four biovars (Hayward, 1964). Subsequently, utilization of trehalose and production of gas from nitrate were added to the above-mentioned biochemical tests. Consequently, the strains were grouped into five biovars and five races (Hayward, 1991). A tropical variant of biovar 2 (2T or NT) has been proposed as a sixth biovar (Fegan *et al.*, 1998a). Comparisons between the race and biovar classification systems of *R. solanacearum* indicate that although the biovar system defines the groups more accurately than the race system, the respective classification systems do not classify *R. solanacearum* into similar groups (Champoiseau *et al.*, 2009). The only overlap between them is that biovar 2 strains often belong to race 3 (Hayward, 1991). One of the crucial shortcomings of the biovar classification system is the lack of well-defined groups, which makes it unsuitable for defining *R. solanacearum* phylogeny (Champoiseau *et al.*, 2009).

R. solanacearum has also been reclassified based on molecular techniques. Restriction fragment length polymorphism (RFLP) analyses were initially used to divide the strains into two major divisions and various sub-clusters or RFLP groups (Cook *et al.*, 1989). Division I encompasses biovars 3, 4 and 5 (isolates from Asia), whilst division II contains biovars 1, 2 and NT (isolates from the Americas) (Cook *et al.*, 1989; Cook and Sequeira, 1994). The existence of these two major genetic clusters has furthermore been confirmed by 16S rDNA sequence analyses (Li *et al.*, 1993; Seal *et al.*, 1993; Taghavi *et al*., 1996). A more recent multilocus sequence typing (MLST) study of *R. solanacearum* strains, based on the internal transcribed spacer region, the endoglucanase (*egl*) gene and the *hrpB* gene, which encodes a regulator of pathogenicity genes, has produced a comprehensive hierarchal-based classification system (Fegan and Prior, 2005). This classification system is comprised of four phylotypes, in which each phylotype corresponds to a unique genetic group, and is further divided into sequence variants (sequevars), which can be resolved further into a number of clonal lines (Fegan and Prior, 2005). Strains in each phylotype appear to have evolved separately due to geographical isolation. Thus, each phylotype reflects the geographic origin of strains: phylotype I is composed of strains from Asia, phylotype II is comprised of strains from America, phylotype III contains strains primarily from Africa and surrounding islands, and phylotype IV has isolates from Indonesia, Japan and Australia. In addition, phylotype IV contains two close relatives of *R. solanacearum*, namely *Ralstonia szygii* and the blood disease bacterium (BDB) (Taghavi *et al.*, 1996). The phylotyping classification scheme reflects clearly the genetic diversity of *R. solanacearum* resulting from geographic separation (Fegan and Prior, 2005) and it can also divide some strains into variable pathogenicity groups (Wicker *et al*., 2007).

The phylotype-based *R. solanacearum* classification system displays significant correlation with other phenotypic and molecular-based classification schemes described previously (Fegan and Prior, 2005). Race 1, defined by its broad host range (Buddenhagen *et al.*, 1962), has strains divided into all four phylotypes described thus far. In contrast, races 2 and 3, known to have very narrow host ranges, have representative strains in at most two phylotypes. Biovars 1 and 2T are present in three of the four phylotypes and consequently, identification of a strain as biovar 1 or 2T appears to be uninformative (Fegan and Prior, 2005). As mentioned earlier, most strains belonging to biovar 2 are equivalent to race 3 (Hayward, 1991) and therefore belong to phylotype II sequevars 1 and 2. However, some biovar 2 strains do not belong to race 3 and are found in phylotype IV sequevars 8 and 9. Moreover, this

phylotypic scheme of classification largely confirms the RFLP typing scheme: phylotype I is equivalent to division I defined by Cook and Sequeira (1994), whereas phylotype II is equivalent to division II of Cook *et al.* (1989). According to Fegan and Prior (2005), phylotypes III and IV were missed completely in the RFLP typing scheme, as well as in 16S rDNA sequence analyses (Cook *et al.*, 1989; Cook and Sequeira, 1994; Li *et al.*, 1993; Seal *et al.*, 1993; Taghavi *et al*., 1996). At the sequevar level, which is below the phylotype level, the RFLP and phylotyping schemes are once again in agreement. Cook *et al.* (1989) described race 2 strains to belong to three different multiple locus genotypes (MLGs). These genetic variations are well captured in the phylotypic classification scheme of *R. solanacearum*, in that each strain in the different MLGs belongs to distinct sequevars (Fegan and Prior, 2005).

1.3 BACTERIAL WILT DISEASE

Irrespective of its classification, *R. solanacearum* is a devastating phytopathogen with a global distribution, and an unusual and ever-increasing host range (Hayward, 1991; Prior *et al.*, 1998; Poussier *et al.*, 2000; Janse *et al.*, 2004). This wide host range covers more than 450 different plant species that include herbaceous plants, shrubs and trees (Swanson *et al*., 2005). The bacterium represents a major economical concern given its ability to seriously affect the production of ornamental plants (Williamson *et al.*, 2002; Swanson *et al.*, 2005) and other valuable crops, such as peanut, eggplant, tomato and potato (Janse *et al*., 2004). Potato brown rot is one of the most destructive vascular diseases of potato. This is due to its aggressiveness, particularly in warm climatic conditions (Poussier *et al.*, 2000). Although it was initially thought to be a threat only in tropical regions, the tomato- and potato-infecting *R. solanacearum* strains have been shown to adapt and survive in cool temperate conditions (Elphinstone *et al*., 1998; Williamson *et al.*, 2002; Janse *et al.*, 2004). *R. solanacearum* race 3 biovar 2 was reported to have an increased host range that includes most solanaceous and non-solanaceous plants. These plants include eggplants, pepper, weeds and geraniums, which serve as alternative hosts. The ability to survive in alternative hosts where there may be only latent infection furthermore complicates management of the disease (Pradhanang *et al.*, 2000; Janse *et al.*, 2004). Consequently, *R. solanacearum* is a zero tolerance quarantine pathogen in most European countries (Swanson *et al.*, 2005). Moreover, increased uncontrollable plant devastation has also led to consideration of this pathogen as a potential select bioterror agent (Young *et al.*, 2008).

1.4 MANAGEMENT OF *R. solanacearum*

Several strategies have been put in place to manage bacterial wilt disease, including crop rotation (Adhikari and Basnyat, 1998), chemical control (Hayward, 1991; Swanson *et al.*, 2005), breeding for resistant cultivars (Lin *et al.*, 2008), use of biological agents (Lopez and Biosca, 2004), as well as various soil amendment approaches (Anith *et al.*, 2004; Dannon and Wydra, 2004; Lin *et al.*, 2008). Unfortunately, all of these have been met with little success. Failure by most of the management approaches is attributed to the wide host range of *R. solanacearum*, the immense genetic variation among strains, as well as its ability to cause latent infections and to survive for long periods in the absence of its host (van Elsas *et al.*, 2000; Janse *et al.*, 2004; Lopez and Biosca, 2004). Although breeding of resistant cultivars is the ideal control strategy for any plant disease, this approach is frustrated by the genetic diversity in virulence of *R. solanacearum* strains and geographic-specific resistance (Lin *et al.*, 2008). Nevertheless, research on the model plant *Arabidopsis thaliana* has indicated that certain ecotypes show resistance to the pathogen. This phenomenon is being exploited towards gaining a better understanding of plant resistance to bacterial wilt disease and subsequent identification of novel resistance genes (Deslandes *et al.*, 1998; 2005). The use of chemical and biological control is hindered by the pathogen's localization within the plant's vascular systems, as well as in deep soil layers. In addition, chemical control could be detrimental to the environment, whilst all of the biological control agents tested thus far were unable to survive under conditions where *R. solanacearum* thrives (Brown and Allen, 2004). A better understanding regarding the pathogenesis of *R. solanacearum* and its ability to survive in different ecological niches would thus be invaluable towards devising a novel, efficient control strategy.

1.5 PATHOGENESIS OF *R. solanacearum*

Although *R. solanacearum* pathogenesis is poorly understood, several virulence factors have been described. These include exopolysaccharide I (EPS I) (McGarvey *et al.*, 1998; 1999), plant cell wall-degrading enzymes and proteins capable of suppressing plant defense mechanisms (Schell, 1987; 2000; Schell *et al.*, 1994; Poueymiro and Genin, 2009). Expression of various virulence factors is under the control of both quorum sensing and phenotype conversion systems (Shinohara *et al.*, 2006). These two systems ensure coordinated expression of virulence genes, thus preventing premature alert of the host plant

defense system when the pathogen inoculum is too low for establishing a productive infection (Gonzalez and Keshaven, 2006). The successful promotion of disease development by bacterial pathogens is not only dependent on expression of virulence factors, but also on their successful delivery into host cells. In *R. solanacearum*, most of the effector proteins described thus far has been shown to be delivered directly into plant tissues by the type III secretion (T3S) system (van Gijsegem *et al.*, 2000). Activation of T3S system genes is entirely dependent on physical contact with plant tissues, since its gene expression is under the influence of a plant-inducible promoter (Lee *et al.*, 2008). Thus, bacterial adherence is critical for disease development as it provides a physical link between the pathogen and the host, which is a prerequisite for the infection process (Henderson *et al.*, 1999).

1.5.1 Infection process of *R. solanacearum*

The invasion of plants by *R. solanacearum* has generally been thought to occur through the roots only, but aerial transmission by insects has been reported for some *R. solanacearum* strains infecting banana (Soguilon *et al.*, 1994; 1995). However, according to Hayward (2005), there is insufficient evidence to support the latter. The current model of the *R. solanacearum* infection process is therefore based on the root infection route. The *R. solanacearum* inoculum in soil can come from various sources, *e.g.* infected potato plants and tubers, as well as latently infected weeds and ornamentals, *e.g.* geranium, which may or may not show any disease symptoms (Janse *et al.*, 2004; Swanson *et al.*, 2005). Infected plants constantly shed bacteria into the soil where they can survive for periods from five to twelve years in the absence of their host. The inoculum can also be from contaminated household or industrial waste water when used for irrigation, or from contaminated farming or packing equipment (Janse, 2006).

According to Vasse *et al*. (1995; 2005), *R. solanacearum* present in the soil invades the vascular tissues of the plant through wounded roots, natural openings that occur at the emergence of secondary roots, or at the root tips. This is followed by colonization of intercellular spaces and disruption of cell walls in the root tissues, thereby facilitating xylem invasion. In the xylem, the bacterial population rapidly multiplies and may reach a density of 10^{10} CFU/ml of xylem fluid (McGarvey *et al.*, 1999). The infection then spreads into upper aerial parts of the plant through the vascular system. Invasion and rapid, systemic colonization require that the pathogen secretes an array of virulence factors, some to its outer

surface, others to the surrounding milieu and still others directly into host plant cells (Schell, 2000). The extensive colonization, coupled with the presence of high quantities of high-molecular-weight extracellular polysaccharides excreted by the pathogen, leads to vascular dysfunction (Kao *et al.*, 1992; Schell, 2000). Water flow is impeded, thus resulting in wilting symptoms, eventual plant collapse and death due to degradation of the vessels and surrounding tissues. Plant collapse and death permit the bacteria to return to the soil where they live a saprophytic life style, whilst awaiting a new host (Kelman and Sequeira, 1965).

The ability of *R. solanacearum* to infect a wide range of plants can be attributed to its expression of an arsenal of different virulence factors. Apart from the aforementioned virulence factors in *R. solanacearum*, many putative virulence genes have been identified following whole-genome sequence analysis (Salanoubat *et al.*, 2002). The genome was found to be organized into two large circular replicons, namely a 3.7-Mb chromosome and a 2.1-Mb megaplasmid. This genomic arrangement is typical for most if not all *Ralstonia* and *Burkholderia* species (Coenye and Vandamme, 2003). The whole-genome sequence analysis confirmed that the megaplasmid harbors many virulence and pathogenicity genes, as previously postulated by Boucher *et al.* (1986). In contrast, the chromosome harbors genes required for normal cellular functions, excepting genes needed for nucleotide synthesis that are only encoded in the megaplasmid. Many genes in the chromosome have been duplicated in the megaplasmid, which has been suggested to be evolving through acquisition of more genes from the chromosome (Salanoubat *et al.*, 2002; Genin and Boucher, 2004).

1.6 BACTERIAL-HOST ADHESION

For bacterial pathogens, recognition of and adherence to host cells are crucial steps during pathogenesis (Henderson *et al.*, 1999). Delivery of virulence factors into host cells is completely dependent on physical contact between the pathogen and the host cell (Henderson *et al.*, 1999). Gram-negative bacteria, including *R. solanacearum*, have a number of surface appendages for adherence (Salanoubat *et al.*, 2002). Certain bacteria can adhere non-specifically to surfaces using non-fimbrial structural adhesins that are present as monomers or oligomers on the outer membrane. In contrast, pathogens typically have one or more extracellular structures for specific adherence to host cells (Marceau *et al.*, 1998; Henderson *et al.*, 1999). Adherence is frequently mediated by flagella, pili and adhesins, as well as

extracellular polymeric substances (O'Toole and Kolter, 1998; DeFlaun *et al.*, 1999; Genevaux *et al.*, 1999; Espinosa-Urgel *et al.*, 2000; Tans-Kersten *et al.*, 2001; Kang *et al.*, 2002; Jackson *et al.*, 2004). Notably, Salanoubat *et al.* (2002) reported that the *R. solanacearum* genome contains a large number of putative genes that code for outer membrane proteins or components of bacterial appendages that may facilitate adherence of the bacterium.

1.6.1 Flagella

Mutagenesis studies have shown that flagella play an important role in bacterial adherence (O'Toole and Kolter, 1998). The absence of flagella in plant-associated bacteria, such as *Pseudomonas fluorescens* and *Pseudomonas putida*, has been reported to result in an impaired ability of these bacteria to colonize potato and wheat roots (De Weger *et al.*, 1987; DeFlaun *et al.*, 1994). During *R. solanacearum* pathogenesis the flagella are proposed to be involved early in wilt disease development. It is thought to play a role in the localization and transport of the bacterium to the host plant, as well as in initial colonization (Tans-Kersten *et al*., 2001). This is based on observations that a non-motile, aflagellate mutant strain of *R. solanacearum* shows reduced virulence in a soil soak assay, which requires the bacteria to actively locate and invade plant roots from the soil. In contrast, when this non-motile aflagellate mutant was applied directly to cut tomato petioles, it was as virulent as the parent strain (Tans-Kersten *et al*., 2001).

1.6.2 Pili and adhesins

Previous studies have shown that pili and pilus-associated adhesins are important for adherence to and colonization of surfaces by bacteria (Pratt and Kolter, 1998; Kang *et al.*, 2002; Black *et al.*, 2006). Pili are hair-like structures that radiate from the bacterial cell surface and may present adhesive moieties, called adhesins, which recognize and bind to specific host cell receptors (Hultgren *et al.*, 1996). This binding event leads either to a commensal or pathogenic relationship with the host. Pili implicated in adherence to biotic and abiotic surfaces include type I pili (Pratt and Kolter, 1998; Eto *et al.*, 2007), P pili (Kuehn *et al*., 1992; Rose *et al*., 2008) and type IV pili (T4P) (Kang *et al*., 2002; Craig *et al*., 2004; Craig and Li, 2008). Amongst these, T4P are well characterized and most widespread, being present in almost all Gram-negative bacteria (Mattick, 2002; Rakotoarivonina *et al*., 2002; Craig *et al*., 2004). Pili have been shown to be an important virulence factor associated with a

wide range of diseases (Hahn, 1997; Tacket *et al.*, 1998). Not only do pili facilitate contact with the host, but they have also been shown to mediate other diverse functions. These include flagella-independent surface translocation (Wall and Kaiser, 1999; Kang *et al.*, 2002), microcolony and biofilm formation (O'Toole and Kolter, 1998), cell signaling (Zhang and Normark, 1996), DNA uptake (Krause *et al.*, 2000; Kang *et al*., 2002), and they can be used as receptors by some bacteriophages (Skerker and Shapiro, 2000).

1.7 PILUS BIOGENESIS AND SECRETION SYSTEMS

Based on the diverse roles of pili, the next part of the literature review will focus on various types of pilus biogenesis systems. This section will be introduced by a brief summary of protein secretion systems, of which some are involved in the assembly and secretion of pili.

Protein secretion does not only involve processes in which a protein crosses the outer membrane barrier into the extracellular milieu, but also the export of proteins that remain anchored to the cell surface such as flagella, pili and pilus-associated adhesins (Thanassi and Hultgren, 2000; Desvaux *et al.*, 2004). Gram-negative bacteria possess at least six different pathways for protein secretion (Fig. 1.1), and their classification is broadly based on sequence, structure and function (Lory, 1998; Thanassi and Hultgren, 2000; Tseng *et al.*, 2009).

In Gram-negative bacteria, some secreted proteins are exported across the inner and outer membranes in a single step. Single-step transfer across the membranes and into eukaryotic cells can occur either via the type I secretion (T1S) system, known to deliver adhesion proteins (Delepelaire, 2004), type III secretion (T3S) system, known to be a hypersensitivity response and pathogenicity determinant (Casper-Lindley *et al.*, 2002; van Gijsegem *et al.*, 2000), or the novel type VI secretion (T6S) system, whose function is aided by both the haemolysin-coregulated protein (Hcp) and Val-Gly Repeats (Vgr) that are thought to bind and/or poke the target host cell (Boyer *et al.*, 2009).

Other proteins are first exported into the periplasmic space via the universal general secretory (Sec) or twin-arginine translocation (Tat) pathways. The proteins are then translocated across the outer membrane via either the type II secretion (T2) system, responsible for secretion of

cell wall-degrading enzymes (Brown and Allen, 2004; Gonzalez *et al.*, 2007), or type V secretion (T5S) system (Mori and Ito, 2001; Tseng *et al.*, 2009). The T5S system, also referred to as the chaperone-usher pathway, is entirely dedicated to the assembly of surface structures in most bacteria (Thanassi *et al.*, 1998; Sauer *et al.*, 2000). Notably, the T3S system may also be involved in the assembly of pili. This is based on results indicating that pili of *Pseudomonas syringae* could not be assembled in a mutant strain harboring a non-functional T3S system (Roine *et al.*, 1997). Remarkably, *R. solanacearum* possesses genetic information for all six major secretion systems reported in Gram-negative bacteria (Salanoubat *et al.*, 2002; Genin and Boucher, 2004).

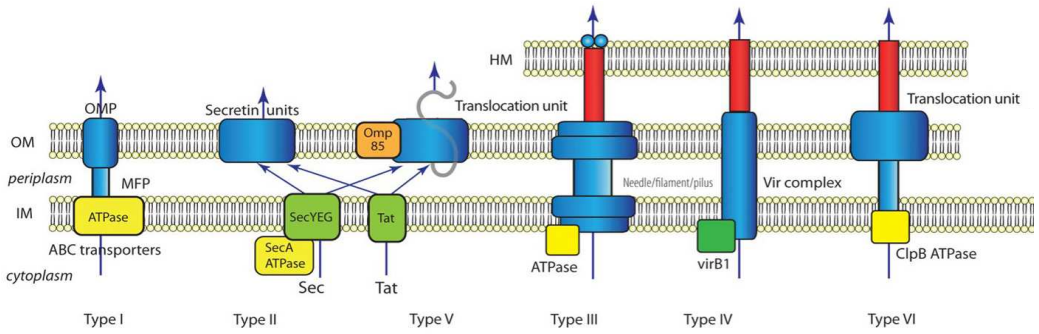


Fig. 1.1 Diagram illustrating the different secretion systems present in Gram-negative bacteria (Tseng *et al.*, 2009). Type II and V secretion systems are dependent on the general secretory pathway (GSP). In contrast, type I, III, IV and VI secretion systems are GSP-independent and are capable of directly translocating their proteins across the inner and outer membranes. HM: host membrane; OM: outer membrane; IM: inner membrane; OMP: outer membrane protein; MFP: membrane fusion protein.

1.7.1 Type IV pili (T4P)

T4P are hair-like appendages that extend from the bacterial cell surface and mediate adherence to various surfaces (Soto and Hultgren, 1999). They are present in human pathogens such as enteropathogenic *E. coli* (EPEC), *P. aeruginosa* and *Vibrio cholerae*, as well as phytopathogens, such as *R. solanacearum* (Farinha *et al.*, 1994; Liu *et al.*, 2001; Kang *et al.*, 2002; Mattick, 2002; Craig *et al.*, 2004). The T4P have been implicated in virulence, DNA uptake, twitching motility and the promotion of interbacterial interactions leading to autoaggregation (biofilm) on host tissues (Craig and Li, 2008).

1.7.1.1 General characteristics of T4P

T4P are identified based on amino acid sequence similarities of the major pilin subunit, as well as morphological traits such as the width, length and flexibility of the pili (Strom and Lory, 1993; Pelicic, 2008). Based on immunologic and crystallographic data, T4P are thin with a diameter of 50-80 Å and are typically up to 4 000 nm long, with a pitch distance of *ca.* 40 Å and about five subunits per turn (Parge *et al.*, 1995). These pili are flexible but strong fibers and many pili interact to form bundles (Craig *et al.*, 2004). A typical T4P is comprised of one major subunit, which possesses a number of unique properties. It contains a short basic amino (N)-terminus leader peptide, a modified amino acid (*N*-methylophenylalanine) at the N-terminus of mature pilin, a highly hydrophobic N-terminal domain, and a disulfide-bonded carboxy (C)-terminal domain (Hobbs and Mattick, 1993; Pugsley, 1993; Alm and Mattick, 1997). The leader peptide is cleaved by a specific/specialized inner membrane signal peptidase during maturation of the pilin subunit (Lory and Strom, 1997). In the majority of bacteria possessing T4P, the major pilins have been implicated in adherence to surfaces (Farinha *et al.*, 1994; Lee *et al.*, 1994).

Although most T4P are flexible structures and arranged in a helical manner, the toxin-coregulated pili (Tcp) of *V. cholerae* (Taylor *et al.*, 1987) and the bundle-forming pili (Bfp) of enteropathogenic *E. coli* (Girón *et al.*, 1991) have some unique characteristics among T4P pili. The TcpA and BfpA pilins assemble into straight fibers of variable length that have a strong tendency to aggregate laterally (Bieber *et al.*, 1998). In addition, whereas most type IV prepilin proteins are characterized by a short basic leader sequence (less than 10 amino acids) and a conserved phenylalanine residue that follows the cleavage site (referred to as T4ap), the TcpA and Bfp prepilins are characterized by larger leader peptides (15-30 amino acids) and the absence of phenylalanine in the position immediately after the conserved leader peptide cleavage site (referred to as T4bp) (Craig *et al*., 2004). Moreover, the mature type IVa pilin sequence is about 150 amino acids in length, whilst the amino acid sequence of the mature type IVb pilin may vary in length from 40-50 to 190 amino acids (Pelicic, 2008).

1.7.1.2 Biogenesis of T4P

Although the genes required for T4ap assembly are typically located in various regions of the bacterial genome (Strom and Lory, 1993; Pelicic, 2008), those required for T4bp assembly are usually clustered together. For example, the *tcp* genes of *V. cholerae* are clustered in a single

region of the chromosome (Kaufman *et al.*, 1993), and the *bfp* genes of enteropathogenic *E. coli* (EPEC) are clustered in an 80-kb virulence plasmid (Girón *et al.*, 1991; Stone *et al.*, 1996). Moreover, in comparison to T4ap, fewer genes are needed for the assembly of T4bp. Nevertheless, certain molecular mechanisms involved in T4ap and T4bp biogenesis are conserved, *e.g.* prepilin transport, processing, filament assembly and pilus emergence on the surface (Pelicic, 2008).

The T4ap biogenesis process appears to require a strikingly large set of dedicated proteins (Martin *et al.*, 1995; Yoshida *et al.*, 1999; Carbonnelle *et al.*, 2005). Characterization of *P. aeruginosa* mutants, which lack twitching motility mediated by T4ap, has led to the identification of several genes involved in T4ap biogenesis and function. These genes are divided into four groups. Firstly, there are four transcriptional regulators (*pilS*, *pilR*, *fimS* and *algR*). The second group comprises of eight *che*-like genes (*pilG*, *H*, *I*, *J*, *K*, *L*, *chpA* and *chpB*), which control T4ap-based twitching motility (Darzins and Russell, 1997). The third group encompasses T4ap biogenesis genes (*pilA*, *B*, *C*, *D*, *E*, *F*, *M*, *N*, *O*, *P*, *Q*, *V*, *W*, *X*, *Y1*, *Y2*, *Z*, *fimT* and *fimU*), and the fourth group has two pilus function genes (*pilT* and *pilU*) (Alm and Mattick, 1997; Wall and Kaiser, 1999).

Recently, Carbonnelle *et al.* (2006) reported that the assembly of T4ap may be much simpler than previously thought (Soto and Hultgren, 1999). In addition to the pilin (PilA), only a core of six proteins (PilD, PilF, PilM, PilN, PilO and PilP) may be required to assemble the prepilin protein into mature T4ap. PilF is an ATPase that provides energy for extrusion of the pili, whilst PilQ, the only protein associated with the outer membrane, is required for the emergence of the fibers on the cell surface. PilD is the prepilin peptidase needed for maturation of the prepilin by cleaving the leader peptide from the prepilin. The PilM, PilN, PilO and PilP proteins might constitute the essence of the machinery necessary for the mechanical assembly of T4ap.

A simplified model for T4ap biogenesis and secretion has been described by Wolfgang *et al.* (2000) and Carbonnelle *et al.* (2006). Following translocation of the pre-PilA precursor subunits into the periplasmic compartment, these subunits are transitorily anchored into the inner membrane at the periplasmic side by a conserved hydrophobic domain located at the N-terminus, immediately after the signal peptide, whilst the hydrophilic C-terminal domains are oriented towards the periplasm (Kaufman *et al.*, 1991; Pugsley, 1996). The hydrophobic N-

terminus is cleaved at the cytoplasmic side of the inner membrane by the prepilin peptidase, PilD, to generate mature PilA (major pilin) (Nunn and Lory, 1991). Finally, the mature pilins pack into a pilus fiber that is exported to the extracellular milieu through a channel formed by the secretin (PilQ) (Collins *et al.*, 2004). The proper localization and stability of the PilQ complex is dependent on an outer membrane lipoprotein (PilP), referred to as a pilotin (Martin *et al.*, 1995; Hardie *et al.*, 1996; Crago and Koronakis, 1998). PilN and PilO form pilus-like structures in the periplasm that guide pilus assembly in-between the membranes (Craig *et al*., 2004). Extrusion and assembly of the pili are facilitated by the energy provided by PilF, which functions as an ATPase (Turner *et al*., 1993; Sakai *et al*., 2001).

1.7.2 Flp pili

In 2000, a novel T4bp biogenesis and secretion system was simultaneously described in *Aggregatibacter actinomycetemcomitans* (formerly known as *Actinobacillus actinomycetemcomitans*) (Kachlany *et al.*, 2000) and *Caulobacter crescentus* (Skerker and Shapiro, 2000). This pilus biogenesis and secretion system is dedicated to the synthesis and secretion of Flp pili (for fimbrial-like protein), a novel and distinct subfamily of T4bp pilin proteins (Kachlany *et al.*, 2000; Perez *et al.*, 2006). Homologues of the Flp pilus biogenesis and secretion system have since been identified in other bacteria and Archaea (Planet *et al.*, 2003; Tomich *et al.*, 2007). The Flp pilus biogenesis and secretion system has been shown to be important for adherence and colonization, and it was also reported to play a role in virulence (Fuller *et al.*, 2000; Nika *et al.*, 2002; Spinola *et al.*, 2003; de Bentzmann *et al.*, 2006; Tomich *et al.*, 2006). The following sections will therefore discuss the Flp pilus biogenesis and secretion system, as described in different bacterial species.

1.7.2.1 The Flp pilus biogenesis and secretion system of *A. actinomycetemcomitans*

A. actinomycetemcomitans is a Gram-negative, facultative anaerobic coccobacillus that inhabits the oral cavities of humans and wild primates (Asikainen *et al.*, 1991; Meyer and Fives-Taylor, 1998; Henderson *et al.*, 2003). It is an opportunistic pathogen and has been implicated as the primary etiological agent of localized juvenile and severe adult periodontitis (Armitage, 1999; Henderson *et al.*, 2003). Moreover, *A. actinomycetemcomitans* has also been associated with non-oral infections, including endocarditis, septicaemia and abscesses (Van Winkelhoff and Slots, 1999).

Fresh clinical isolates of *A. actinomycetemcomitans* are able to adhere tightly to solid abiotic surfaces, such as glass, plastic and hydroxyapatite, and are capable of forming a tenacious biofilm (Scannapieco *et al.*, 1983; 1987; Inouye *et al.*, 1990; Fine *et al.*, 1999a; Kachlany *et al.*, 2000). Electron microscopy studies of the clinical isolates revealed that they express long, thick fibrils (Fives-Taylor *et al.*, 2000; Kachlany *et al.*, 2000). Each fibril consists of a parallel array of individual pili of *ca.* 5 to 7 nm in diameter, and the fibrils become interlocked by sharing individual pili (Inouye *et al.*, 1990; Kachlany *et al.*, 2001). The fibrils are often several microns long and up to 100 nm thick (Kachlany *et al.*, 2001). On solid medium, colonies of fresh clinical isolates display rough colony morphology (Inouye *et al.*, 1990; Fine *et al.*, 1999b). However, these colonies convert to a smooth phenotype upon repeated subculture and this conversion is accompanied by a loss of fimbriae and autoaggregation (Inouye *et al.*, 1990; Fine *et al.*, 1999b; Slots and Ting, 1999; Kachlany *et al.*, 2000). These findings suggested that the fimbriae (pili), subsequently termed Flp pili, are directly responsible for both adherence and autoaggregation of *A. actinomycetemcomitans.*

1.7.2.2 The *tad* locus of *A. actinomycetemcomitans*

Biochemical analysis demonstrated that the Flp pili of *A. actinomycetemcomitans* are composed of a 6.5-kDa polypeptide (Inoue *et al.*, 1998). Using the N-terminal amino acid sequence of the major protein isolated from purified fibril preparations, Inoue *et al.* (1998) identified the nucleotide sequence of the *flp-1* gene, which encodes the major structural component of Flp pili (Kachlany *et al.*, 2001). Subsequently, the genetic locus responsible for the biogenesis and secretion of Flp pili has been identified. This locus, designated the *tad* locus (for tight adherence), comprises 14 genes (*flp-1-flp-2-tadV-rcpCAB-tadZABCDEFG*) (Kachlany *et al.*, 2000), and is presented graphically in Fig. 1.2.

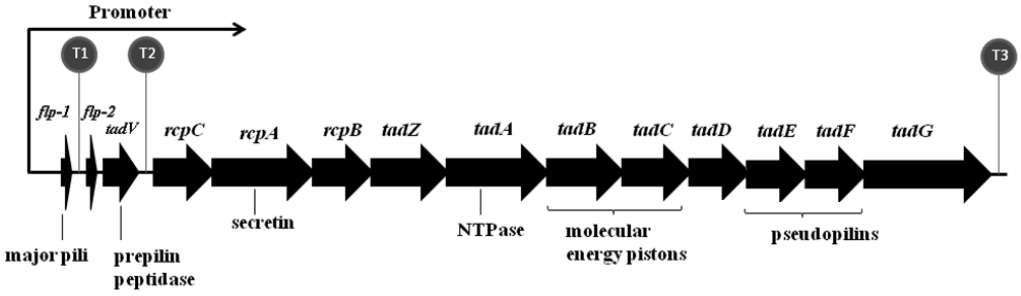


Fig. 1.2. The *tad* locus of *A. actinomycetemcomitans*. The arrows point to the direction of transcription and transcriptional terminators (T1, T2 and T3) are shown by lollipops. T1 and T3 are intrinsic terminators, whilst T2 is a Rho-dependent terminator (Modified from Kram *et al.*, 2008).

- ***flp* and *tadV* genes**

The *flp-1* gene, which encodes the Flp prepilin protein, is located immediately downstream of the promoter region. The Flp1 precursor has a signal peptide sequence at its N-terminus that is cleaved upon export from the bacterial cell (Tomich *et al.*, 2006). Immediately downstream of *flp-1* is a second *flp* allele, designated *flp-2*. Phylogenetic analysis has indicated that not only is Flp2 a homologue of Flp1, but also that both Flp1 and Flp2 belong to a distinct subfamily of the T4b pilin proteins (Kachlany *et al.*, 2001). Although insertion mutagenesis studies have indicated that *flp-1* mutants fail to adhere to surfaces and do not express pili, the functional significance of *flp-2* is not known as the gene does not appear to be expressed in *A. actinomycetemcomitans* (Kachlany *et al.*, 2001; Perez *et al.*, 2006).

The *tadV* gene of *A. actinomycetemcomitans* is located between the *flp* and *rcpCAB* genes, and encodes a prepilin peptidase with homology to those of T4P and T2S systems (Tomich *et al*., 2006). It was subsequently shown that the TadV protein is required and sufficient for maturation of the Flp pili, as well as for maturation of TadE and TadF pseudopilin proteins (Tomich *et al.*, 2006). Although the TadV protein contains two highly conserved aspartate residues that are critical for proteolysis, it lacks the N-terminal methyltransferase domains found in T4P and T2S prepilin peptidases. Therefore, it was proposed that TadV may be a novel non-methylating aspartic acid prepilin peptidase (Tomich *et al.*, 2006).

- ***rcp* genes**

Immediately downstream of the *tadV* gene are the *rcpCAB* (for rough colony protein) genes, which were initially identified in a study by Haase *et al.* (1999). This study compared protein profiles of rough and smooth strains of *A. actinomycetemcomitans*. The results indicated that the RcpA and RcpB proteins are present in outer membrane protein preparations of rough *A. actinomycetemcomitans* strains only, and entirely absent from smooth non-adherent strains. The RcpA protein is a member of the GspD/PulD family of outer membrane proteins, also referred to as secretins, and is predicted to form the outer membrane channel for secretion of the Flp pili (Kachlany *et al*., 2001; Clock *et al.*, 2008). In contrast, the RcpB protein has no known homologues in other bacterial secretion systems. The role of the RcpB protein is unclear, because gene inactivation studies have failed to produce viable mutants or a functional Tad biogenesis system (Wang and Chen, 2005; Perez *et al.*, 2006). Although inactivation of *rcpB* has been achieved in smooth non-piliated strains, such inactivation is not

useful since the *tad* genes are either poorly expressed or not expressed at all in these strains (Kachlany *et al.*, 2000; Perez *et al.*, 2006). Nevertheless, since RcpB was exclusively isolated in outer membrane protein preparations of *A. actinomycetemcomitans*, it was proposed that this protein may be required for the assembly and/or stability of the secretin complex. Alternatively, it was suggested to gate the secretin pore, thus ensuring the integrity of the outer membrane during pilin extrusion (Perez *et al.*, 2006). Similar to RcpB, the RcpC protein has no homology with known proteins. However, it is speculated that this protein may be involved in post-translational modification of the Flp pili by glycosylation (Tomich *et al.*, 2006). This is based on the observation that the Flp protein of an *rcpC* mutant migrates faster through a SDS-polyacrylamide gel compared to the Flp protein of the parent strain. Iyer and Aravind (2004) identified two tandem β-clip domains in RcpC, which are known to facilitate glycosylation and interaction with carbohydrate moieties. Therefore, it may be possible that one or both of the RcpC β-clip domains interact with the carbohydrates on glycosylated Flp pili to facilitate its extrusion through the secretin ring (Tomich *et al.*, 2007).

- ***tad* genes**

Downstream of *rcpCAB* is a cluster of eight tandem genes (*tadZABCDEFG*), collectively termed the *tad* genes (Kachlany *et al.*, 2000). Mutations in any of the *tad* genes resulted in a defect in adherence and production of Flp pili (Kachlany *et al.*, 2000; Wang and Chen, 2005).

The TadZ protein shows homology with the MinD/ParA superfamily of proteins (Tomich *et al.*, 2007). Whereas MinD has a role in cell division (de Boer *et al.*, 1991; Hu and Lutkenhaus, 1999; Raskin and de Boer, 1999), ParA directs directional chromosome segregation during cell division (Davis *et al.*, 1992). Both MinD and ParA therefore controls distinct cellular localization patterns. The homology of TadZ to this superfamily of proteins has thus been proposed to indicate that it may play a role in localizing the Tad apparatus to the cellular poles of the bacterium (Tomich *et al.*, 2007).

The TadA protein shows homology with secretion NTPases of T4P and T2S systems (Bhattacharjee *et al.*, 2001; Planet *et al.*, 2001). According to Crowther *et al.* (2005), NTPases couple the energy of ATP hydrolysis to protein secretion and/or pilus assembly. Purified TadA of *A. actinomycetemcomitans* has demonstrable ATPase activity and mutagenesis of the Walker box, a nucleotide binding motif in NTPases (Walker *et al.*, 1982), abolished this

activity and Flp pili production (Bhattacharjee *et al.*, 2001). TadA therefore appears to function as an ATPase that provides energy for Flp pilus assembly and, as such, is critical to the functioning of the Flp biogenesis and secretion system (Bhattacharjee *et al.*, 2001).

The TadB and TadC proteins of *A. actinomycetemcomitans* show limited identity (13%) at the amino-acid-level and thus, their genes may be paralogues (Planet *et al.*, 2003). Both proteins exhibit sequence similarity to the PilC family of inner membrane proteins found in T4P and T2S systems (Crowther *et al.*, 2005; Craig *et al.*, 2004). Other known members of the PilC family are the enteropathogenic *E. coli* proteins, BfpE and BfpD. Protein BfpE has been shown to bind to protein BfpD, the cognate ATPase, thus resulting in an increase in its catalytic activity (Crowther *et al.*, 2005). BfpD serves as a motor that generates energy through ATP hydrolysis and transfers it to the BfpE protein, which, in turn, channels it into pilus elongation (Craig *et al.*, 2004; Crowther *et al.*, 2005). Therefore, similar to BfpD and BfpE, the TadB and TadC proteins are proposed to function as molecular pistons that channel the energy of ATP hydrolysis from TadA into Flp pilus polymerization. Alternatively, it was also proposed that they may serve as a membrane support of the pilus biogenesis apparatus at the inner membrane (Tomich *et al.*, 2007).

The TadD protein lacks homology with proteins present in other bacterial secretion systems, such as the T4P and T2S systems. Nevertheless, the TadD protein is a predicted lipoprotein that is localized to the outer membrane. The protein has a tetratricopeptide repeat (TPR) domain, which is required for protein-protein interactions, and it was suggested that TadD is crucial for ensuring proper assembly and stability of the RcpA secretin (Clock *et al.*, 2008).

The TadE and TadF proteins are termed pseudopilins since they exhibit pilus-like features, but do not appear to be assembled into the Flp pili (Tomich *et al.*, 2006). The TadE and TadF proteins are 22% identical at the amino-acid-level, and their genes are likely to be paralogues (Tomich *et al.*, 2006). Both these proteins have an N-terminal signal peptide and the sequence of the processing site is similar to that of the Flp prepilin protein (Tomich *et al.*, 2006). The TadE and TadF pseudopilin proteins appear to interact and are both essential for Flp pilus biogenesis. This is based on the observation that overexpression of either pseudopilin does not restore Flp pilus biogenesis in the absence of the other pseudopilin (Tomich *et al.*, 2006). Analogous to pseudopilins of T2S systems, the TadE and TadF proteins are proposed to form

pilus-like structures in the periplasm that may aid in the extrusion of the Flp pili (Tomich *et al.*, 2007).

The TadG protein was initially identified in Flp pilus preparations and thought to be a structural subunit of Flp pili (Inouye *et al.*, 1990; Inoue *et al.*, 1998), before the identification of Flp as the major pilin (Kachlany *et al.*, 2001). No homologues of TadG have been identified in any of the known bacterial secretion systems (Kachlany *et al*., 2000). However, Wang and Chen (2005) suggested that TadG may be responsible for anchoring of the Flp pilus to the cell. This is based on the presence of a von Willebrand factor A (VWA) domain that has several functions, including mediation of protein-protein interactions (Whittaker and Hynes, 2002). It has more recently been proposed that the TadG VWA domain may rather be involved in protein-protein interactions with other Tad components (Tomich *et al.*, 2007). Nevertheless, TadG appears to be indispensable for Flp pilin biogenesis as a *tadG* mutant is non-piliated (Kachlany *et al*., 2000).

1.7.2.3 Regulation of the *tad* locus of *A. actinomycetemcomitans*

As indicated above, the function of some of the Tad proteins have been elucidated in various studies. However, little is known regarding the operation and regulation of the *tad* locus in *A. actinomycetemcomitans*, and no specific environmental cues have been shown to trigger transcription of the *tad* locus (Clock *et al.*, 2008). Transcription of the *tad* locus appears to be controlled from a central position, as a single very strong σ^{70} promoter is located upstream of the *flp-1* gene (Fig. 1.2) (Haase *et al.*, 2003; Wang and Chen, 2005; Kram *et al.*, 2008).

Recently, Kram *et al.* (2008) reported that a transcriptional termination cascade may be responsible for regulating the relative transcription levels of the *tad* locus genes. Three transcriptional terminators (T1-T3) were identified, two of which (T1 and T3) are likely to be intrinsic terminators and the third (T2) appears to act through a Rho-dependent mechanism. The T1 and T2 terminators are located in the intergenic region between the *flp-1* and *flp-2* genes and between the *tadV* and *rcpC* genes, respectively, whereas the T3 terminator is located immediately downstream of the *tadG* gene (Fig. 1.2). It was suggested that the differential expression of genes in the *tad* locus of *A. actinomycetemcomitans* can be achieved through termination of transcription at the different terminators, thereby yielding different abundances in the transcripts that correlate with the estimated stoichiometry of the Tad

proteins required for efficient pilus biogenesis (Inoue *et al.*, 1998; Bhattacharjee *et al.*, 1998; Tomich *et al.*, 2006). Based on this model, it therefore follows that expression of the *tad* genes from the three different polycistronic mRNA transcripts would yield a high abundance of Flp1 and slightly less of the TadV prepilin peptidase, whilst proteins that compose the structural components of the Tad machinery would likely be present in small amounts (Kram *et al.*, 2008). It should, however, be noted that the predicted 12-kb transcript corresponding to the entire *tad* locus has not yet been detected. This may be due to rapid processing events or the transcript may be highly unstable (Kram *et al.*, 2008).

1.7.3 The Flp pilus biogenesis and secretion system of *Haemophilus ducreyi*

H. ducreyi is the etiological agent of the sexually transmitted disease chancroid (Morse, 1989). Nika *et al.* (2002) identified a 15-gene cluster in the genome of *H. ducreyi* that encodes proteins with significant homology to those encoded by the *A. actinomycetemcomitans tad* locus. Three *flp* alleles were identified in the *H. ducreyi* gene cluster. Whereas *flp-1* encodes a protein that is 46% identical to Flp1 of *A. actinomycetemcomitans*, the *flp-2* and *flp-3* alleles encode proteins more similar to Flp1 of *H. ducreyi* than to Flp1 of *A. actinomycetemcomitans* (88% and 50%, respectively). Mutations within the *flp-1* and *flp-2* genes of *H. ducreyi* significantly reduced the ability of the bacteria to adhere to a plastic surface and to form microcolonies when cultured *in vitro* with human foreskin fibroblasts. Microcolony formation does not appear to be essential for virulence, since *H. ducreyi* mutants with an in-frame perturbation of the *flp* gene cluster, despite resulting in a deficiency in microcolony formation, were fully virulent in a rabbit model for experimental chancroid (Nika *et al.*, 2002). However, a mutant *H. ducreyi* strain with an inactivated *tadA* gene exhibited decreased virulence in this rabbit model (Nika *et al.*, 2002).

1.7.4 The Flp pilus biogenesis and secretion system of *Pseudomonas aeruginosa*

P. aeruginosa is a ubiquitous Gram-negative bacterium and is known to be highly successful in colonizing diverse environments (Palleroni, 1992a; 1992b). The bacterium is an opportunistic pathogen of humans, causing infection in immunocompromised patients (Van Delden and Iglewski, 1998; Ramsey and Wozniak, 2005). *In silico* analysis of the available genome sequence of *P. aeruginosa* led to the identification of sequences that are homologous to several of the previously reported *flp*, *rcp* and *tad* genes (van Schalkwyk, 2003). This has subsequently been confirmed by de Bentzmann *et al.* (2006). It was furthermore shown that

they encode a functional system for the assembly of Flp pili at the cell surface, since inactivation of the *tadA* and *rcpA*-like genes in *P. aeruginosa* resulted in cells with no observable Flp pili (de Bentzmann *et al.*, 2006). Notably, the *flp* and *rcp-tad* genes are divergently orientated, whilst the *rcpB* and *tadE* genes are not readily identifiable in the *tad* gene cluster of *P. aeruginosa* (de Bentzmann *et al.*, 2006). Furthermore, the *P. aeruginosa tad* gene cluster contains three genes (PA4298, *pprA* and *pprB*) that appear to be unique to *P. aeruginosa* (de Bentzmann *et al.*, 2006).

Interestingly, the *P. aeruginosa tad* gene cluster has a unique genetic organization. The gene cluster is organized into five transcriptional units that are divergently transcribed (Fig. 1.3) (Bernard *et al.*, 2009). The transcriptional units are comprised of the gene encoding Flp pili, nine genes (*rcpC-tadG*) that constitute the *rcp-tad* locus, the gene encoding the response regulator PprB, the gene encoding the FppA prepilin peptidase, and the genes encoding a pseudopilin (*tadF*) and a sensor kinase (*pprA*). In *P. aeruginosa*, global approaches have shown that some *tad* genes are either regulated by quorum sensing (Schuster *et al.*, 2003; Wagner *et al.*, 2003), or are under the control of the VqsR master regulator (Juhas *et al.*, 2005) or the MvaT transcriptional regulator (Vallet *et al.*, 2004). More recently, Bernard *et al.* (2009) reported that the *flp* gene is expressed late in the stationary growth phase in aerobic conditions and that *tad* gene expression was positively controlled by the PprB response regulator. The PprB response regulator was shown to bind to the respective promoter regions, directly controlling the expression of these genes.

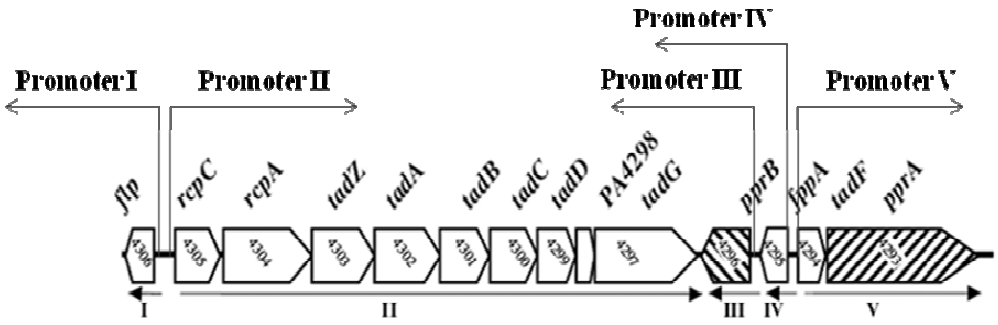


Fig. 1.3 The *tad* locus of *P. aeruginosa*, showing the putative location of each *tad* gene and the various promoters (I-V). The five promoter regions have binding sites for the response regulatory protein PprB. The arrows point to the direction of transcription of the respective transcriptional units (Modified from Bernard *et al*., 2009).

1.7.5 The PilA-Cpa pilus system of *Caulobacter crescentus*

A seven-gene *pilA-cpa* locus (for *Caulobacter* pilus assembly) in *C. crescentus*, a non-pathogenic α-proteobacterium, was found to be responsible for the production of pili of unknown function, although it appears that the pili are used as receptors by bacteriophage ΦCbK (Skerker and Shapiro, 2000). The *C. crescentus* pilus-encoding region contains the pilin gene *pilA*, which is a member of the Flp subfamily of T4b pilin proteins, as well as several homologues of genes present in the *tad* locus of *A. actinomycetemcomitans* (Skerker and Shapiro, 2000). Transcriptional regulation of *pilA* was shown to be under the control of a two-component response regulator responsible for cell cycle control, namely CtrA (Skerker and Shapiro, 2000). In addition, the transcription profile of all the genes in the *pilA-cpa* gene cluster of *C. crescentus* has been determined using DNA microarray analysis. The genes *cpaB-cpaF* are co-induced 15 min before the *pilA* gene, and the gene encoding the putative prepilin peptidase, *cpaA*, is induced slightly after the *cpaB-cpaF* group. These genes are thus all induced prior to pilin gene transcription and pilus assembly, suggesting that may function in pilus biogenesis and secretion (Skerker and Shapiro, 2000).

1.8 AIMS OF THIS STUDY

Bacterial wilt, of which *R. solanacearum* is the etiological agent, is a destructive plant disease worldwide (Hayward, 1991; Prior *et al*., 1998; Poussier *et al*., 2000; Janse *et al*., 2004). Although many different methods for managing the disease have been reported, most of them are ineffective. Failure of most methods has been ascribed to the ever-increasing host range of *R. solanacearum*, its ability to form latent infections, and its ability to survive for long periods in water and soil in the absence of host plants (Elphinstone *et al*., 1998; van Elsas *et al*., 2001; Williamson *et al*., 2002; Janse *et al*., 2004). It can be envisaged that investigations into the pathogenesis and survival strategies of *R. solanacearum* in diverse niches may help to overcome these impediments. A crucial step for disease development is the adherence of *R. solanacearum* to its plant host (Genin and Boucher, 2004), and both flagella and T4ap have been implicated in this process (Liu *et al*., 2001; Tans-Kersten *et al*., 2001; Kang *et al*., 2002). However, Kang *et al*. (2002) reported that although a *pilA* mutant of *R. solanacearum* lacked T4ap, it was still able to adhere to tobacco suspension cells and to tomato roots. Moreover, the mutant strain was also capable of causing wilt disease in both young and mature tomato plants, albeit slower than the parent strain in mature plants (Kang *et al*., 2002). This therefore

suggests the presence of as yet unidentified structures that may influence the adherence and virulence properties of *R. solanacearum*. Subsequent to the above-mentioned studies in *R. solanacearum*, a novel T4bp pilus biogenesis and secretion system involved in adherence and colonization has been described in *A. actinomycetemcomitans* (Kachlany *et al.*, 2000), and homologues of this system have since been identified in the genomes of a wide variety of Gram-negative and Gram-positive bacteria (Planet *et al.*, 2003; Tomich *et al.*, 2007). Notably, it has also been reported that the novel Flp pilus assembly and secretion system plays a role in virulence of *A. actinomycetemcomitans* (Schreiner *et al*., 2003), *H. ducreyi* (Nika *et al.*, 2002; Spinola *et al.*, 2003) and *Pasteurella multocida* (Fuller *et al*., 2000).

Therefore, based on the above, the objectives of this study were:

- To identify homologues in *R. solanacearum* of genes previously described to compose the Flp pilus biogenesis and secretion system, through *in silico* analysis of the available genome sequence.

- To investigate the importance of the Flp pilus biogenesis and secretion system in *R. solanacearum* virulence on potato, through the generation of an appropriate mutant strain and comparison of the strain to the parent strain with regards to its ability to induce disease on potato.

CHAPTER TWO

IN SILICO IDENTIFICATION AND CHARACTERIZATION OF FLP PILUS BIOGENESIS AND SECRETION SYSTEMS IN *Ralstonia solanacearum*

2.1 INTRODUCTION

Bacterial wilt, of which *Ralstonia solanacearum* is the causal agent, is an economically important plant disease globally (Poussier *et al.*, 2000; Swanson *et al.*, 2005). Despite the devastation caused by the disease, little progress toward controlling this phytopathogen has been made. Management of bacterial wilt is complicated by, amongst other, increased resistance of *R. solanacearum* to various antibiotics and metals (Mergeay *et al*., 2003; Swanson *et al.*, 2005), an ever-widening plant host range (Williamson *et al.*, 2002; Janse *et al.*, 2004), and its ability to adapt and thrive in diverse environmental niches (Elphinstone *et al*., 1998; Pradhanang *et al.*, 2000; van Elsas *et al.*, 2000; Janse *et al.*, 2004). In addition to its many secreted virulence factors (Schell *et al.*, 1994; McGarvey *et al.*, 1999; Gonzalez *et al.*, 2007), *R. solanacearum* is also capable of readily acquiring novel genes, possibly through horizontal gene transfer (Sarkar and Guttman, 2004) or through modification of pre-existing genes (Lavie *et al*., 2004; Genin and Boucher, 2004). The observed genetic versatility thus also contributes to the success of *R. solanacearum* to grow and survive in different ecological niches, as well as to cause infections in diverse plant species (Salanoubat *et al.*, 2002).

The complete sequence of the *R. solanacearum* GMI1000 genome, comprising a chromosome and megaplasmid, has been determined (Salanoubat *et al.*, 2002). Whole-genome sequence analysis indicated that not only are many genes in the chromosome duplicated in the megaplasmid, but also that the megaplasmid harbors a considerable number of virulence and pathogenicity genes (Salanoubat *et al.*, 2002; Genin and Boucher, 2004). The ability of phytopathogens, including *R. solanacearum*, to adhere to plant tissues is a crucial first step in the pathogenesis process (Henderson *et al*., 1999). In this regard, it is particularly interesting to note that *R. solanacearum* encodes a large number of putative proteins that may aid adherence of the bacterium to surfaces (Salanoubat *et al.*, 2002). Using a reverse genetics approach, both flagella and type IVa pili (T4ap) have been implicated in *R. solanacearum* adherence and virulence (Liu *et al*., 2001; Tans-Kersten *et al.*, 2001; Kang *et al.*, 2002). The results, however, do not exclude the involvement of other as yet uncharacterized surface appendages, since a mutant strain of *R. solanacearum* deficient in T4ap was reported to be capable of both adhering to plant cells and causing wilt disease (Kang *et al.*, 2002).

Research performed during the last decade has led to the identification and characterization of a novel class of type IVb pili (T4bp) (Kachlany *et al.*, 2000). These pili, commonly referred to as Flp pili, have been identified in human pathogenic bacteria such as *Aggregatibacter actinomycetemcomitans* (Kachlany *et al.*, 2000), *Haemophilus ducreyi* (Nika *et al.*, 2002) and *Pseudomonas aeruginosa* (van Schalkwyk, 2003; de Bentzmann *et al.*, 2006). The *flp* gene, which encodes the major fimbrial subunit, proved to be part of a large gene cluster, termed the *tad* locus, which encodes a secretion system for the assembly and release of the pili (Kachlany *et al.*, 2000; de Bentzmann *et al.*, 2006). It has been reported that the *tad* locus is required by *A. actinomycetemcomitans* and *P. aeruginosa* for adherence since cells containing mutations in this gene cluster failed to adhere to surfaces (Kachlany *et al.*, 2001; de Bentzmann *et al.*, 2006). Moreover, it has been reported that mutations in the *tadA* gene, which encodes a secretion ATPase, significantly reduced virulence by *A. actinomycetemcomitans* and *H. ducreyi* (Schreiner *et al.*, 2003; Spinola *et al.*, 2003). Although homologues of the Flp pilus biogenesis and secretion system have since been identified in the genomes of a wide variety of Gram-negative and Gram-positive bacteria (Planet *et al.*, 2003; Tomich *et al.*, 2007), the best studied Tad systems are those of *A. actinomycetemcomitans* and *P. aeruginosa*.

Since Flp pili have been implicated in adherence and virulence, the aim of this part of the study was therefore to determine whether *R. solanacearum* possesses a pilus biogenesis and secretion system similar to that described above. This was investigated by searching for homologues of the *tad* locus genes in the *R. solanacearum* genome sequence, followed by sequence analysis of the putative *R. solanacearum* proteins to identify conserved features that may be related to their function.

2.2 MATERIALS AND METHODS

2.2.1 Computational analyses

Nucleotide sequences of *R. solanacearum* potentially coding for proteins similar to the *flp*, *rcp* and *tad* gene products of *A. actinomycetemcomitans* and *P. aeruginosa* were identified using the BLAST program to search the *R. solanacearum* GMI1000 genome sequence (available at http://sequence.toulouse.inra.fr/R.solanacearum.html). Amino acid sequences of the putative *R. solanacearum* proteins were then compared to entries of the GenBank database by making use of the BLAST-P program (Altschul *et al.*, 1997) available on the National

Centre for Biotechnology Information web page (http://www.ncbi.nlm.nih.gov/BLAST/). Default settings of the BLAST program were used and the names of previously identified sequences obtained from these searches were retained in this study. Pair-wise alignments were performed with BLAST-P, while multiple alignments were carried out with MAFFT v.6 (Katoh *et al.*, 2002). Phylogenetic analyses were conducted with MEGA v.4 (Tamura *et al.*, 2007), and Neighbor-Joining (NJ) distance-based phylogenetic trees were constructed (Saitou and Nei, 1987). Branch support was estimated with non-parametric bootstrap analysis based on 1000 replicates (Felsenstein, 1985).

The amino acid sequence of individual *R. solanacearum* proteins was analyzed with different programs, as indicated in Table 2.1, to gain a better understanding regarding their possible function. Conserved motifs or domains were identified with the SMART tool, signal peptide prediction was performed with SIGNALP and the cellular location of proteins was predicted with PSORT. Domains typical of cell membrane-associated proteins were identified with DAS, whilst transmembrane domains were predicted with TMPRED. Lipoproteins were identified with LipoP.

Table 2.1 Programs used to identify different features in *R. solanacearum* proteins

Program	Uniform Resource Locator (URL) address	Reference
SMART tool	http://smart.embl-heidelberg.de/smart	Letunic *et al.* (2009)
SIGNALP	http://www.cbs.dtu.dk/services/SignalP	Bendtsen *et al.*, (2004)
PSORT	http://psort.ims.u-tokyo.ac.jp	Nakai and Kanehisa (1991)
DAS	http://www.sbc.su.se/~miklos/DAS	Cserzo *et al.* (1997)
TMPRED	http://www.ch.embnet.org/software/TMPRED	Hofmann and Stoffel (1993)
LipoP	http://www.cbs.dtu.dk/services/LipoP	Rahman *et al.* (2008)

The G+C content of *R. solanacearum* genomic regions was calculated with the DNA/RNA GC Content Calculator (at http://www.endmemo.com/bio/gc.php), whilst the theoretical molecular mass (Mw) of the different proteins was determined with the pI/Mw tool (at http://ca.expasy.org/tools/pi_tool.html). Searches for promoter sequences and transcription start sites were performed with BPROM tools (at http://www.softberry.com/berry.html) and SAK software (Gordon *et al.*, 2003).

2.3 RESULTS

2.3.1 Identification of gene clusters in *R. solanacearum* homologous to the *tad* loci of different bacteria

BLAST searches of the *R. solanacearum* genome with each of the proteins encoded by the *tad* gene clusters in *A. actinomycetemcomitans* and *P. aeruginosa*, respectively, led to the identification of two *tad* loci (Fig. 2.1). The first of these is present in a 12.633-kb region of the *R. solanacearum* megaplasmid, located at nucleotides 137 0181-138 2814, and contains 14 open reading frames (RSP1079 through RSP1092). Although these ORFs encode predicted proteins with homology to most of the known Tad system proteins, homologues of a second Flp prepilin protein, as well as TadE and RcpB proteins encoded by the *tad* locus of *A. actinomycetemcomitans* could not be identified. These proteins are also absent from the Tad system of *P. aeruginosa*. Moreover, protein RSP1081 displayed amino acid sequence identity with PA4298 of *P. aeruginosa*. This protein has thus far been reported only in the *tad* gene cluster of *P. aeruginosa*, but its function is not yet known (de Bentzmann *et al.*, 2006). Proteins RSP1079 and RSP1088 appear to be unique to the *tad* locus in the megaplasmid, although protein RSP1079 displayed amino acid sequence identity with the transcriptional regulator PilR (Darzins and Russell, 1997). The G+C content of genes comprising the megaplasmid *tad* locus ranged between 61% (RSP1092) and 74% (RSP1088) and the locus has an average G+C content of 69.5%, which is similar to the average G+C content of the megaplasmid (66.8%) (Salanoubat *et al.*, 2002).

The second *tad* locus is present in a 11.156-kb region of the *R. solanacearum* chromosome, located at nucleotides 695 339-706 494, and also contains 14 ORFs (RSC0648 through RSC0661). Despite the presence of a similar number of ORFs, this *tad* locus differs from that in the megaplasmid in that it encodes four putative Flp prepilin proteins and lacks homologues to three of the proteins encoded by the megaplasmid *tad* locus (*i.e.* RSP1079, RSP1081 and RSP1088). Moreover, the chromosomal *tad* locus does not encode a homologue of the RcpB protein of *A. actinomycetemcomitans*, and appears to also lack homologues of the TadD and TadF proteins. The chromosomal *tad* locus encodes for a protein, RSC0654, which does not display homology with any known proteins, but appears to possess properties of a lipoprotein. Examination of the G+C content of genes comprising the chromosomal *tad* locus indicated that it ranged between 60% (RSC0660 and RSC0661) and 72% (RSC0658) with an average G+C content of 67.6%, which is similar to the average G+C content of the chromosome (67.04%) (Salanoubat *et al.*, 2002).

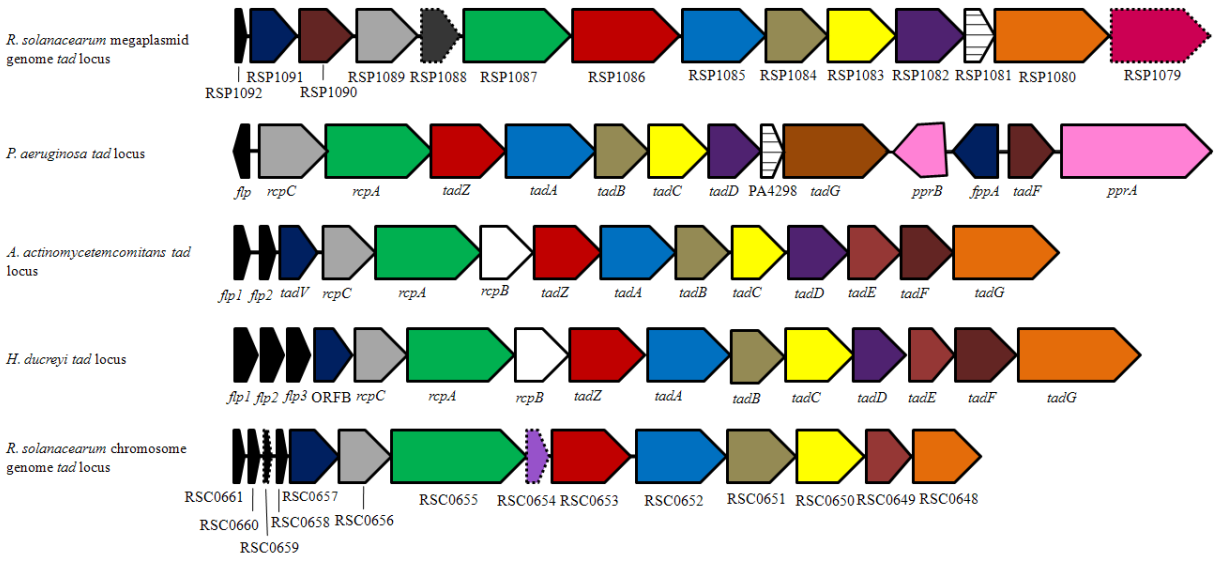


Fig. 2.1 Organization of the megaplasmid and chromosomal *tad* gene clusters in *R. solanacearum* compared to the homologous gene clusters of *P. aeruginosa* (Bernard *et al*., 2009), *A. actinomycetemcomitans* (Kram *et al*., 2008) and *H. ducreyi* (Tomich *et al*., 2007). ORFs with similar predicted protein products (as determined by BLAST analyses) are indicated in the same colour. 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* chromosomal *tad* locus are indicated by a dashed outline.

3.3.2 Identification of putative consensus promoter sequences

To identify consensus promoter sequences the genomic sequence of *R. solanacearum* encompassing the megaplasmid and chromosomal *tad* loci, together with up- and downstream flanking sequences, were analyzed with different programs. These analyses indicated the presence of two different promoters in regions upstream of the respective *tad* gene clusters only (Fig. 2.2). The sequence of the putative promoters upstream of the *tad* locus in the megaplasmid corresponded to that of a sigma 70 (σ^{70})-dependent promoter (TTGACT-14 bp-TTGAAT) and a σ^{54}-dependent promoter (GGCGGTTTTCGTGC). Similarly, the region upstream of the chromosomal *tad* locus was predicted to contain a σ^{70}-dependent promoter (TTGGCC-18 bp-TTAAAT) and a σ^{54}-dependent promoter (GGTGAAGTCGGGGGAGC).

In contrast to σ^{70}, which is involved in expression of most genes during exponential growth (Lonetto *et al.*, 1992), σ^{54} appears to play a role in many different biological activities across the eubacteria (Merrick, 1993; Barrios *et al.*, 1999). Among the proteobacteria, the functions carried out by the products of σ^{54}-dependent transcription include utilization of various nitrogen and carbon sources, energy metabolism, chemotaxis and flagellation (Merrick, 1993). In *P. aeruginosa*, σ^{54} is important, amongst other, for flagella and pilus synthesis (Ishimoto and Lory, 1989; Totten *et al.*, 1990). The lack of any obvious theme in the diverse functions carried out by products of σ^{54}-dependent transcription has been suggested to be indicative of σ^{54} being biologically important and advantageous (Barrios *et al.*, 1999). A review of the *R. solanacearum* GMI1000 genome sequence identified two genes, annotated as *rpoN1* (RSC0408 in the chromosome) and *rpoN2* (RSP1671 in the megaplasmid), which encode homologues of σ^{54}. The *R. solanacearum* σ^{54} putative proteins display 43% amino acid sequence identity to each other, and RpoN1 and RpoN2 are, respectively, 45% and 38% identical to σ^{54} of *P. aeruginosa*.

(a)

(b)

Fig. 2.2 Sequences of the predicted promoters located upstream of the megaplasmid (a) and chromosomal (b) *tad* gene clusters of *R. solanacearum*. The predicted -10 and -35 hexamers of σ^{70}-dependent promoters are shown in black and grey boxes, respectively, and the predicted transcriptional start sites are shown in large font. The predicted σ^{54}-dependent promoters are underlined and indicated in bold letters. The arrows indicate the direction of transcription, starting with the first gene of each gene cluster.

2.3.3 *In silico* characterization of proteins encoded by the *tad* loci in *R. solanacearum*

To detect homologues of the predicted protein products encoded by the *R. solanacearum tad* loci, each of the different proteins were compared to proteins in the GenBank database using the BLAST-P tool (Altschul *et al.*, 1997). The proteins were also analyzed with various different programs, as indicated under Materials and Methods, to gain a better understanding regarding their possible function(s). The results of these analyses for the predicted proteins encoded by the *tad* loci in the megaplasmid and chromosome of *R. solanacearum* are summarized in Tables 2.2 and 2.3, respectively.

2.3.3.1 Flp pilin and pilin-like proteins

Homologues of the Flp prepilin protein

The precursor of the major structural component of Flp pili is encoded by the *flp-1* gene of *A. actinomycetemcomitans* (Inoue *et al.*, 1998; Kachlany *et al.*, 2001) and *P. aeruginosa* (de Bentzmann *et al.*, 2006). However, in *tad* loci, genes encoding Flp subunits may also be found closely linked to other probable *flp* alleles (Tomich *et al.*, 2007). For example, the genomes of *H. ducreyi* (Nika *et al.*, 2002) contain three clustered *flp* alleles, whereas *A. actinomycetemcomitans* has two alleles (Kachlany *et al.*, 2001), but *P. aeruginosa* has a single allele (de Bentzmann *et al.*, 2006).

Homology searches indicated that protein RSP1092, encoded by the *tad* locus in the megaplasmid, displays amino acid sequence identity with the Flp1 and Flp2 proteins of *A. actinomycetemcomitans* (51% and 52% sequence identity, respectively). Likewise, proteins RSC0658, RSC0659, RSC0660 and RSC0661, encoded by the *tad* locus in the chromosome, are all predicted to be Flp prepilin proteins. Although proteins RSC0658, RSC0660 and RSC0661 display homology with the Flp protein of *P. aeruginosa* (34%, 23% and 27% amino acid sequence identity, respectively), RSC0658 is the only protein that displays significant amino acid sequence identity with the Flp protein of *A. actinomycetemcomitans* (30% sequence identity with Flp1).

Members of the Flp subfamily of T4b pilin proteins are readily identifiable by the presence of a Flp motif (G-XXXXEY) at the amino (N)-terminus of the mature protein, which occurs within a stretch of *ca.* 20 hydrophobic non-polar, aliphatic amino acids. It has been shown that the Flp prepilin protein is cleaved at a site following the glycine (G) residue in the above

consensus sequence (Inoue *et al.*, 1998; Kachlany *et al.*, 2001; Tomich *et al.*, 2006). In addition, members of the subfamily usually contain a phenylalanine (F) residue close to the middle of the hydrophilic carboxy (C)-terminus (Kachlany *et al.*, 2001; Tomich *et al.*, 2006). Analysis of the different Flp putative proteins in *R. solanacearum* indicated that only the megaplasmid-encoded protein RSP1092 contained all of these features. In contrast, the chromosomally encoded Flp putative proteins all lacked a phenylalanine residue in the C-terminus, whilst protein RSC0659 lacked the Flp motif (Fig. 2.3a). Interestingly, in contrast to the megaplasmid-encoded Flp putative protein (RSP1092), those encoded by the chromosome clustered as a distinct group within the Flp subfamily of T4b pilin proteins (Fig. 2.3b).

Analysis of protein RSP1092 indicated that it consists of 58 amino acid residues and has an estimated molecular mass of 5.9 kDa. The protein contains a Flp processing motif, as indicated above, with the cleavage site between Gly_{14} and Ala_{15} in the sequence $_{10}$RDEQG↓ATAIEY$_{20}$, thus yielding a mature protein with a molecular mass of 4.3 kDa. Analysis of proteins RSC0658, RSC0660 and RSC0661, each of which is comprised of 53 amino acid residues and has an estimated molecular mass of 5.7 kDa, indicated that cleavage of the Flp processing site between Gly_{14} and Ala_{15} in the sequences $_{10}$RNEDG↓AASTEY$_{20}$ (RSC0658) or $_{10}$REEDG↓AAGVEY$_{20}$ (RSC0660 and RSC0661) would yield mature proteins of 4.1 kDa.

Homologues of Tad pseudopilin proteins

The *tad* locus of *P. aeruginosa* encodes a single pseudopilin protein (TadF) (de Bentzmann *et al.*, 2006), whilst that of *A. actinomycetemcomitans* encodes two pseudopilin proteins (TadE and TadF) (Kachlany *et al.*, 2000). In contrast to *P. aeruginosa* (Bernard *et al.*, 2009), it has been reported that both pseudopilin proteins are essential for Flp biogenesis in *A. actinomycetemcomitans* (Kachlany *et al.*, 2000; Tomich *et al.*, 2006). Although the mechanism by which these pseudopilin proteins function in *A. actinomycetemcomitans* Flp assembly and secretion is not clear, Tomich *et al.* (2007) proposed that they may function in a manner analogous to that described for pseudopilins of type II secretion (T2S) systems. The latter pseudopilins are thought to form a pilus-like structure in the periplasmic space, which may function as a piston that extrudes the secretion substrate through the outer membrane (Sandkvist, 2001).

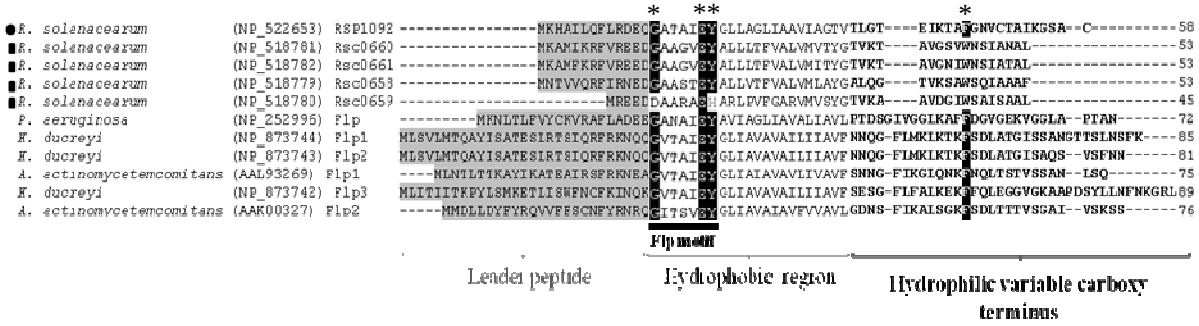


Fig. 2.3a Alignment of the putative Flp proteins of *R. solanacearum* with several proteins of the Flp subfamily of T4b pilin proteins. The Flp prepillin proteins comprise of three domains, *i.e.* a leader peptide, hydrophobic region and hydrophilic variable C-terminus, and contain a conserved Flp motif (underlined). 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 prepillin protein encoded by the megaplasmid *tad* locus of *R. solanacearum* is indicated by a circle, whilst those encoded by the chromosomal *tad* locus are indicated by squares. For all sequences included in the analysis, the GenBank accession numbers are indicated in brackets.

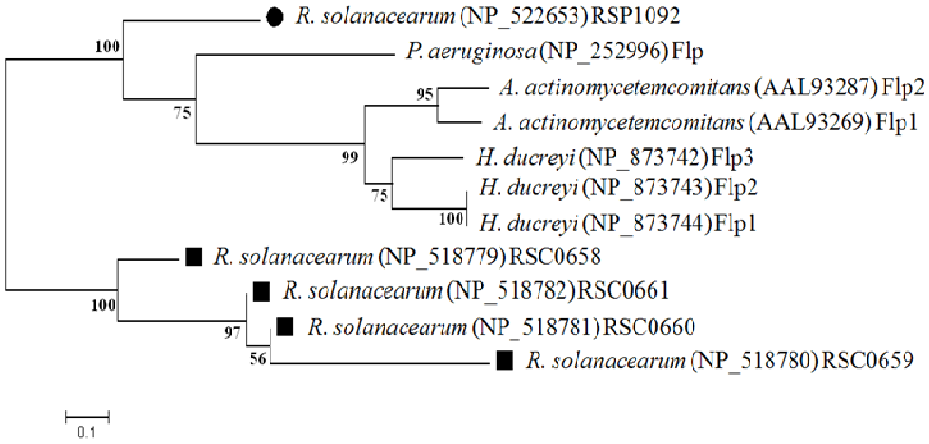


Fig. 2.3b Phylogeny of Flp proteins. The tree was generated with Neighbor-Joining distance analysis, and bootstrap support values are indicated at the internodes. The putative Flp prepillin protein encoded by the megaplasmid *tad* locus of *R. solanacearum* is indicated by a circle, whilst those encoded by the chromosomal *tad* locus are indicated by squares. GenBank sequence accession numbers are indicated in brackets.

Each of the *tad* loci in *R. solanacearum* encodes a single protein that displays homology with previously described pseudopilin proteins. The megaplasmid-encoded protein RSP1090 displays significant amino acid sequence identity with the TadF protein of *P. aeruginosa* only (33% sequence identity). Whether the chromosome-encoded protein RSC0649 could be related to TadE or TadF is unclear, as it displays 30% amino acid sequence identity with the TadE protein of *A. actinomycetemcomitans* and 37% amino acid sequence identity with the TadF protein of *P. aeruginosa*.

The domain architectures of pseudopilin proteins exhibit striking similarity to not only each other, but also to that of the Flp prepillin protein (Tomich *et al.*, 2006). Both pseudopilin and Flp proteins contain a N-terminal signal sequence, followed by a hydrophobic domain. In the case of pseudopilin proteins, the N-terminal signal sequence is flanked by a highly conserved sequence, G-XXXXEF/L, which strongly resembles the Flp motif in the prepillin proteins (G-XXXXEY) (Tomich *et al.*, 2006). In the case of *A. actinomycetemcomitans*, it has been reported that the TadE and TadF pseudopilin proteins are both processed by the TadV prepilin peptidase after the glycine (G) residues within their N-termini, and the processing step appears to be crucial for the functions of the TadE and TadF proteins (Tomich *et al.*, 2006). Analysis of the *R. solanacearum* proteins RSP1090 (163 amino acid residues, 15.2 kDa) and RSC0649 (144 amino acid residues, 17 kDa) indicated that they share several of these features common to pseudopillin proteins. Proteins RSP1090 and RSC0649 possess three and two transmembrane domains, respectively, and are both predicted to be localized to the inner membrane. Both these proteins also contain the consensus pseudopilin processing site (Fig. 2.4) with the cleavage site predicted to be between Gly_{28} and Val_{29} in the sequence $_{24}$RGCRG↓VAAVEY$_{34}$ of protein RSP1090, and between Gly_{15} and Thr_{16} in the sequence $_{11}$RRMAG↓TAAVEY$_{21}$ of protein RSC0649. Cleavage at these sites would yield mature proteins of 13.5 and 13.9 kDa, respectively.

2.3.3.2 Prepilin peptidase

Homologues of Flp prepilin peptidases

For pili to be assembled, a peptidase is required that processes the signal peptide in the prepilin protein (Christie, 1997; Soto and Hultgren, 1999). In *A. actinomycetemcomitans*, the TadV protein has been reported to be both required and sufficient for maturation of the Flp pilin and pseudopilin proteins (Tomich *et al.*, 2006). Likewise, the FppA protein of

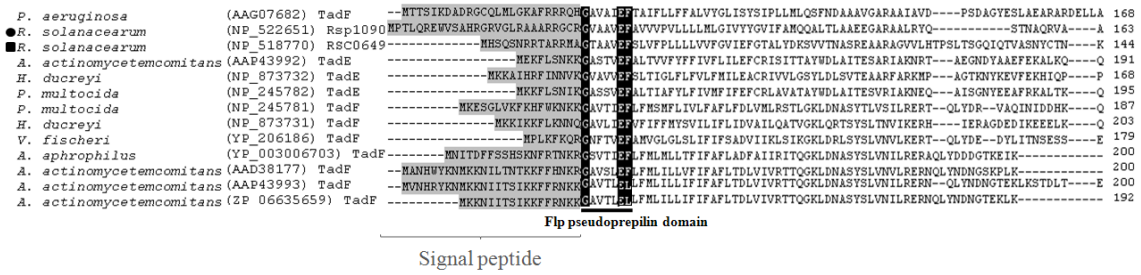


Fig. 2.4 Alignment of the putative pseudopilin proteins of *R. solanacearum* with those encoded by other Tad systems. The leader peptide sequences are shown in grey and the region, indicating the conserved sequence present in pseudopilin proteins, is underlined. The putative pseudopilin protein encoded by the megaplasmid *tad* locus of *R. solanacearum* is indicated by a circle, whilst that encoded by the chromosomal *tad* locus is indicated by a square. For all sequences included in the analysis, the GenBank accession numbers are indicated in brackets.

P. aeruginosa, a homologue of TadV, has also been reported to be required for Flp prepilin processing (de Bentzmann *et al.*, 2006).

Homology searches indicated that the *R. solanacearum* proteins RSP1091 and RSC0657, encoded by the *tad* locus in the megaplasmid and chromosome, respectively, exhibit homology with the prepillin peptidases FppA of *P. aeruginosa* (36% and 25% amino acid sequence identity, respectively) and TadV of *A. actinomycetemcomitans* (33% amino acid sequence identity in both cases).

Analysis of proteins RSP1091 (168 amino acid residues, 17.8 kDa) and RSC0657 (172 amino acid residues, 17.3 kDa) indicated that they both lack an apparent signal peptide sequence, possess four transmembrane domains and are predicted to be localized to the inner membrane. BLAST-P searches indicated that these *R. solanacearum* proteins display homology with the prepilin peptidases and signal peptidases of the PilD-GspO/PulO group, which are respectively required for T4ap biogenesis (PilD) (Strom *et al.*, 1993; Pepe and Lory, 1998) and proteolytic processing of proteins that are secreted by T2S systems (GspO/PulO) (Strom *et al.*, 1993; Francetic and Pugsley, 1996; Paetzel *et al.*, 2002). Some of the prepilin peptidases of this group are bifunctional since they have, in addition to peptidase activity, also *N*-methyltransferase activity, which is responsible for *N*-methylation of +1 residues of mature T4a pilins and pseudopilins of T2S systems (Strom *et al.*, 1993; Paetzel *et al.*, 2002). In contrast to the above-mentioned prepilin peptidases, TadV and its homologues in other Tad systems lack N-terminal transferase domains and is thus proposed to form a novel subclass of non-methylating prepilin peptidases (de Bentzmann *et al.*, 2006, Tomich *et al.*, 2006). Comparison of the *R. solanacearum* proteins to the PilD prepilin peptidase of *P. aeruginosa* indicated that both proteins RSP1091 and RSC0657 lack the putative methyltransferase box, LGGKCS, described as being essential for the *N*-methyltransferase activity of PilD (Pepe and Lory, 1998). Moreover, both *R. solanacearum* proteins each contain two aspartic acid-containing domains (Fig. 2.5a) that correspond to the consensus sequences, D(I/L)XXRXL and (G/A)(G/A)GDXKL, reported for Flp prepilin peptidases (Tomich *et al.*, 2006). The aspartic acid residues (D) in these consensus sequences were reported to be critical for prepilin peptidase-dependent maturation of Flp pili (de Bentzmann *et al.*, 2006; Tomich *et al.*, 2006). Phylogenetic analysis, however, revealed that protein RSP1091, in contrast to protein RSC0657, groups closely with the TadV/FppA prepilin peptidases required exclusively for proteolytic maturation of Flp pili and Tad pseudopilin proteins (Fig. 2.5b).

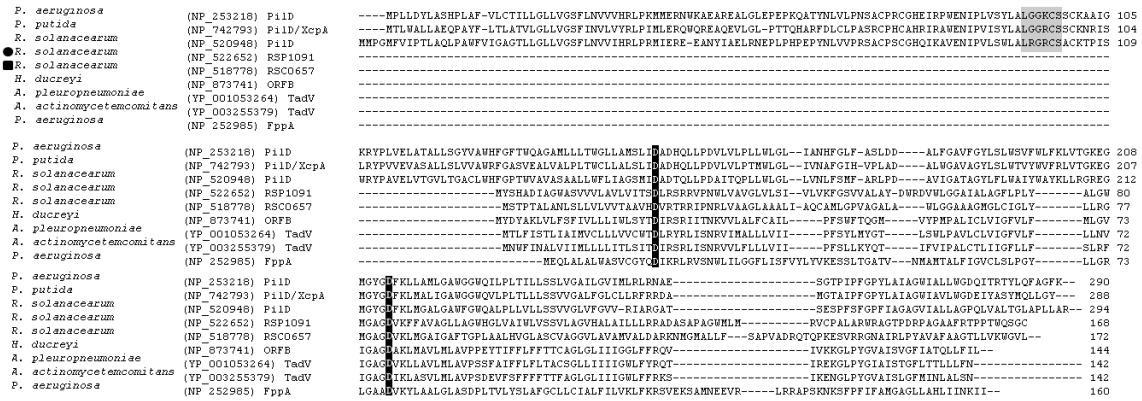


Fig. 2.5a Alignment of the putative prepilin peptidases of *R. solanacearum* with those encoded by other Tad systems, as well as the PilD prepilin peptidase. The conserved aspartic acid (D) sequence present in the respective prepilin peptidases, needed for catalytic activity, is shown in black. The methyltransferase box only present in PilD is shown in grey. The putative signal peptidase protein encoded by the megaplasmid *tad* locus of *R. solanacearum* is indicated by a circle, whilst that encoded by the chromosomal *tad* locus is indicated by a square. For all sequences included in the analysis, the GenBank accession numbers are indicated in brackets.

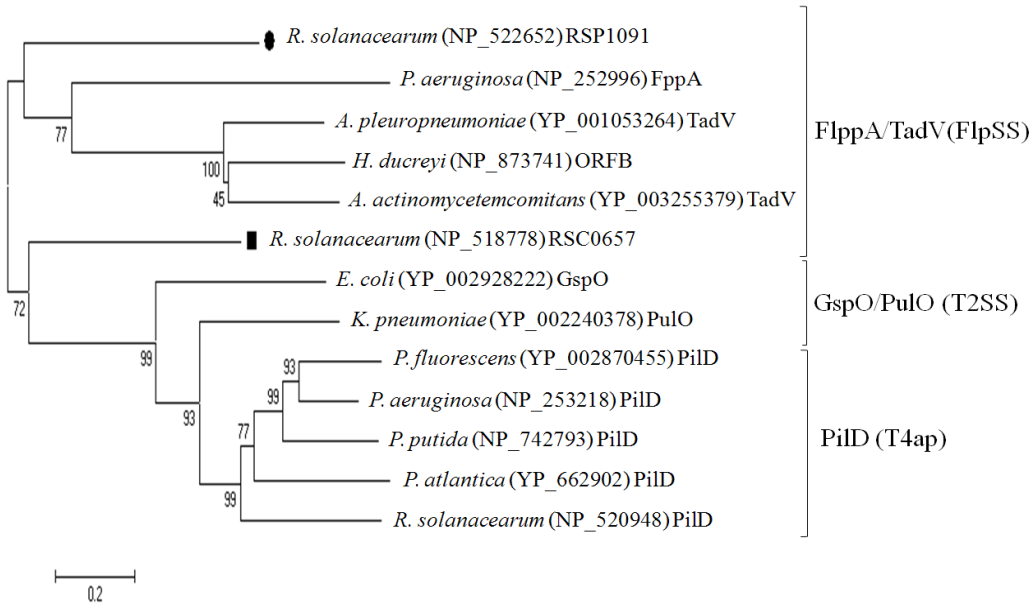


Fig. 2.5b Phylogeny of prepillin peptidases. The tree was generated with Neighbor-Joining distance analysis, and bootstrap support values are indicated at the internodes. The putative Flp prepillin signal peptidase encoded by the megaplasmid *tad* locus of *R. solanacearum* is indicated by a circle, whilst that encoded by the chromosomal *tad* locus is indicated by a square. GenBank sequence accession numbers are indicated in brackets. Abbreviations: FlpSS, Flp pilus secretion system; T2SS, Type II secretion system; T4ap, Type IVa pili.

2.3.3.3 Cytoplasmic proteins

Homologues to TadZ

The TadZ protein is a unique component of the Tad systems, and is found in almost all bacterial *tad* loci, except those from Archaea (Tomich *et al.*, 2007). Although the function of TadZ has not yet been elucidated, it is proposed to play a role in cellular localization of the Tad apparatus (Tomich *et al.*, 2007).

Homology searches indicated that the *R. solanacearum* proteins RSP1086 and RSC0653, encoded by the megaplasmid and chromosomal *tad* loci, respectively, display homology with the TadZ proteins of both *P. aeruginosa* and *A. actinomycetemcomitans*. Protein RSP1086 displays 28% and 21% amino acid sequence identity with TadZ proteins of *P. aeruginosa* and *A. actinomycetemcomitans*, respectively, whilst protein RSC0653 displays 22% and 20% amino acid sequence identity with the TadZ proteins of the respective bacteria.

Proteins RSP1086 (439 amino acid residues, 47.5 kDa) and RSC0653 (397 amino acid residues, 43 kDa) both lack an apparent signal peptide sequence and are predicted to localize to the cytoplasm, although they may be peripherally associated with the inner membrane by means of a single transmembrane domain. Based on sequence comparisons, the TadZ protein of *A. actinomycetemcomitans* has been reported to be a member of the MinD/ParA superfamily of proteins (Tomich *et al.*, 2007). In this regard, it is noteworthy that both RSP1086 and RSC0653 display amino acid sequence identity to MinD, a probable septum site determining protein, of *Rhizobium leguminosarum* (GenBank accession no. YP_002277926.1, 23% and 41% sequence identity, respectively). Cell division follows chromosome replication and involves separation of the daughter chromosomes and movement to the centre of the prospective daughter cells (partitioning), followed by septum formation and cell separation (Schmid and von Freiesleben, 1996). These functions are performed, amongst other, by ParA and MinD, respectively (Davis *et al.*, 1992; Raskin and de Boer, 1999). This therefore suggests that the production of the Flp pili might be linked to cell shape and/or division. Such a link has been suggested for *Caulobacter crescentus* and it was reported that transcription of the *pilA* gene, a homologue of *flp-1*, is cell cycle-regulated and occurs only in late predivisional cells (Skerker and Shapiro, 2000). Moreover, it was subsequently shown that the TadZ homologue CpaE of *C. crescentus* is localized to the pole of the bacterial cells and plays a role in localizing the Tad apparatus to this location (Viollier *et al.*, 2002). Based on the

above, it is tempting to speculate that the *R. solanacearum* proteins RSC0653 and RSP1086 may play a role in guiding placement of the Tad apparatus to the poles of bacterial cells.

Homologues to the TadA secretion NTPase

All known secretion systems have at least one protein that is thought to use NTP hydrolysis to provide energy for secretion complex assembly or the movement of macromolecules across membranes (Thanassi and Hultgren, 2000; Christie, 2001). The TadA protein of *A. actinomycetemcomitans* has been reported to have ATPase activity and is required to energize the assembly or secretion of Flp pili for tight non-specific adherence of the bacteria to surfaces (Bhattacharjee *et al.*, 2001). Likewise, inactivation of the TadA proteins of *H. ducreyi* (Nika *et al.*, 2002) and *P. aeruginosa* (de Bentzmann *et al.*, 2006) was shown to greatly reduce the ability of the bacteria to adhere to abiotic surfaces. Notably, *tadA* mutants of all three these bacteria were reported to lack Flp pili on the surface of the bacterial cells. Interestingly, inactivation of the TadA proteins of *A. actinomycetemcomitans* (Schreiner *et al.*, 2003) and *H. ducreyi* (Spinola *et al.*, 2003) has been reported to result in bacterial strains attenuated for virulence.

Homology searches indicated that the *R. solanacearum* proteins RSP1085, encoded by the megaplasmid *tad* locus, and RSC0652, encoded by the chromosomal *tad* locus, display significant amino acid sequence identity with the TadA proteins of *P. aeruginosa* (49% and 51% sequence identity, respectively) and *A. actinomycetemcomitans* (48% and 46% sequence identity, respectively).

Proteins RSP1085 (439 amino acids, 49.4 kDa) and RSC0652 (397 amino acid residues, 43 kDa) both lack a putative signal peptide sequence and are predicted to be localized to the cytoplasm. However, both proteins contain a single transmembrane domain, indicating that they may also be peripherally associated with the inner membrane. Analysis of the amino acid sequences indicated that both proteins contain four conserved domains present in all secretion NTPases (Fig. 2.6a). These comprise two canonical nucleotide-binding motifs, designated as Walker boxes A and B, and two conserved regions, designated as the Asp and His boxes (Walker *et al.*, 1982; Whitchurch *et al.*, 1991; Possot and Pugsley, 1994), suggesting that nucleotide binding and/or hydrolysis may be critical to the function of these *R. solancearum* proteins. Notably, BLAST-P searches revealed that proteins RSP1085 and RSC0652 display

significant sequence identity to secretion NTPases that belong to the TrbB/VirB11 family of proteins, which are involved in DNA uptake, extracellular secretion and pilus assembly (Hobbs and Mattick, 1993; Christie, 1997). Phylogenetic analysis not only confirmed this close relationship between the proteins, but also indicated that the NTPases of different Tad systems, including proteins RSP1085 and RSC0652 of *R. solancearum*, form a distinct clade (Fig. 2.6b).

2.3.3.4 Inner membrane proteins

Homologues to TadB and TadC

Protein secretion systems require the concerted activities of different proteins to produce energy that is amplified and converted to a mechanical force in order to push the secreted proteins across the cell membranes (Crowther *et al.*, 2005). It has been proposed that TadB and TadC may function as molecular pistons that channel the energy of ATP hydrolysis from TadA into Flp pilus polymerization or, alternatively, they might serve as an inner membrane scaffold for the assembly of the pilus biogenesis apparatus (Tomich *et al.*, 2007).

Proteins RSP1084 and RSC0651, encoded by the *R. solanacearum* megaplasmid and chromosomal *tad* loci, respectively, display amino acid sequence identity with the TadB proteins of *P. aeruginosa* (33% and 27% sequence identity, respectively) and *A. actinomycetemcomitans* (26% and 22% sequence identity, respectively). The downstream proteins RSP1083 and RSC0650 show homology with the TadC proteins of *P. aeruginosa* (32% and 26% sequence identity, respectively) and *A. actinomycetemcomitans* (22% and 23% sequence identity, respectively).

In addition to the pseudopilin proteins and prepilin signal peptidases discussed earlier, all four of the above *R. solanacearum* proteins are predicted to be localized to the inner membrane. The TadB homologues, proteins RSP1084 (308 amino acid residues, 33.9 kDa) and RSC0651 (321 amino acid residues, 36 kDa), are both predicted to possess five transmembrane domains. Whereas the first 25 amino acids of the RSP1084 sequence possess characteristics of a signal peptide, with the cleavage site predicted to be between Ala_{24} and Glu_{25} in the sequence $_{20}$LMLWA↓EAVRR$_{29}$, the first 18 amino acids of the RSC0651 sequence possess characteristics of a signal peptide, with the cleavage site predicted to be between Ala_{17} and

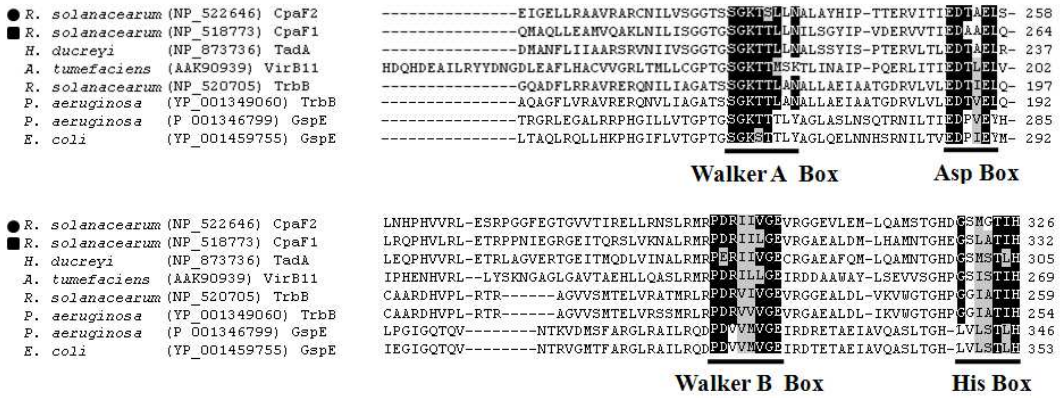


Fig. 2.6a Alignment of the putative TadA proteins of *R. solanacearum* with several members of the TrbB/VirB11 family of secretion NTPases. Only the region of the proteins containing conserved Walker A and B boxes, as well as the consensus Aspartic acid (Asp) and Histidine (His) boxes are shown in the alignment. The putative secretion NTPase encoded by the megaplasmid *tad* locus of *R. solanacearum* is indicated by a circle, whilst that encoded by the chromosomal *tad* locus is indicated by a square.

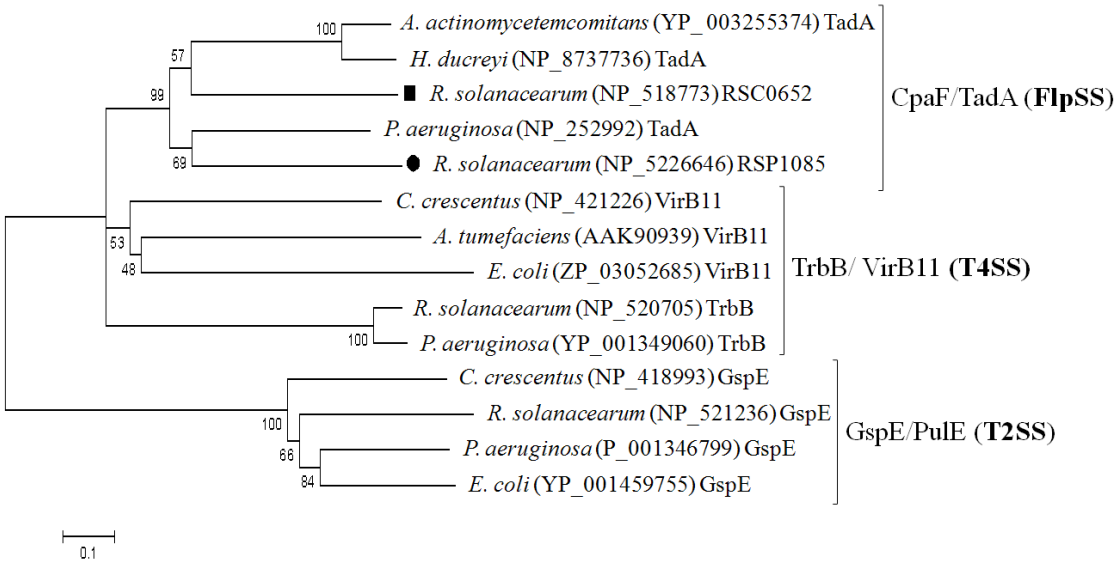


Fig. 2.6b Phylogeny of secretion NTPases. The tree was generated with Neighbor-Joining distance analysis, and bootstrap support values are indicated at the internodes. The putative NTPase encoded by the megaplasmid *tad* locus of *R. solanacearum* is indicated by a circle, whilst that encoded by the chromosomal *tad* locus is indicated by a square. GenBank sequence accession numbers are indicated in brackets. Abbreviations: FlpSS, Flp pilus secretion system; T4SS, Type IV secretion system; T2SS, Type II secretion system.

Leu$_{18}$ in the sequence $_{13}$FVAIA↓LGVLG$_{22}$. Cleavage at these sites would yield mature proteins of 31.4 and 34.1 kDa, respectively. The TadC homologues, proteins RSP1083 (326 amino acids, 35.9 kDa) and RSC0650 (315 amino acids, 34.8 kDa), possess three and four transmembrane domains, respectively. The first 28 amino acids of the RSP1083 sequence possess characteristics of a signal peptide, with the cleavage site predicted to be between Ala$_{27}$ and Val$_{28}$ in the sequence $_{23}$IAVHA↓VLMRH$_{32}$. Cleavage at this site would yield a mature protein of 33.3 kDa. Likewise, protein RSC0650 possess a signal peptide with the cleavage site predicted to between Ala$_{22}$ and Ala$_{23}$ in the sequence $_{18}$GAVLA↓ALTLL$_{27}$, thus yielding a mature protein of 32.6 kDa. Notably, all four of the *R. solanaceaum* proteins contain a conserved protein F domain. Proteins with this domain have been reported to form a platform for the T2S machinery, as well as the type IV pili and the archaeal flagellae (Py *et al.*, 2001). Consequently, it is tempting to speculate that these proteins may play a role in anchoring of the *R. solanacearum* Tad system to the inner membrane.

Homologues to TadG

The TadG protein was initially identified in Flp pilus preparations of *A. actinomycetemcomitans* and was thus proposed to be a structural subunit of Flp pili (Inouye *et al.*, 1990). However, it was more recently proposed that the TadG protein may function to anchor the Flp pilus to the cell (Wang and Chen, 2005). Nevertheless, TadG is reported to be essential for Flp pilus assembly in *A. actinomycetemcomitans* since a *tadG* mutant was either non-piliated (Kachlany *et al.*, 2000) or displayed a reduction in Flp pilus biogenesis (Wang and Chen, 2005).

Homology searches indicated that proteins RSP1080 and RSC0648, encoded by the *tad* loci in the megaplasmid and chromosome, respectively, displayed significant amino acid sequence identity with the TadG protein of *P. aeruginosa* only. Proteins RSP1080 and RSC0648 display 37% and 40% amino acid sequence identity, respectively, with the TadG protein.

Proteins RSP1080 (541 amino acid residues, 49.5 kDa) and RSC0648 (347 amino acid residues, 35.4 kDa) are both predicted to be localized to the inner membrane and posses nine and three transmembrane domains, respectively. The first 25 amino acids of protein RSP1080 possess characteristics of a signal peptide sequence, with the cleavage site between Gly$_{24}$ and Asn$_{25}$ in the sequence $_{20}$SVDIG↓NVFFT$_{29}$, thus yielding a mature protein of 48.2 kDa.

Protein RSC0648 is also predicted to have a N-terminal signal sequence with cleavage occurring between Ala_{36} and Val_{37} in the sequence $_{32}$VGALA↓VDVAR$_{41}$, to yield a mature protein of 33.3 kDa. The TadG proteins have been suggested to facilitate protein-protein interactions with other Tad components through the presence of a von Willebrand factor A (VWA) domain in the TadG protein of *A. actinomycetemcomitans* (Wang and Chen, 2005) or leucine zipper motifs in the TadG protein of *P. aeruginosa* (van Schalkwyk, 2003). However, no similar domains or motifs could be identified in proteins RSP1080 or RSC0648 of *R. solanacearum*. In this regard, it is interesting to note that Tomich *et al.* (2007) reported that the VWA domain appears to be restricted to the TadG proteins of the Pasteurellaceae, the *Vibrionaceae* and *Yersinia* spp., indicating that the domain may have a specialized function in these species.

2.3.3.5 Outer membrane proteins

Homologues to the RcpA secretin

The RcpA protein has been identified in outer membrane preperations of *A. actinomycetemcomitans* (Haase *et al.*, 1999; Clock *et al.*, 2008). Based on its presence in the outer membrane the RcpA protein was proposed to form the channel through which Flp pili traverse the outer membrane (Kachlany *et al.*, 2001). Although the function of RcpA proteins as secretins has yet to be confirmed, it was nevertheless shown that a lack of expression of the RcpA protein in *A. actinomycetemcomitans* is associated with little or no expression of Flp pili (Haase *et al.*, 1999).

The *R. solanacearum* proteins RSP1087 and RSC0655, encoded by the megaplasmid and chromosomal *tad* loci, respectively, exhibit homology with the RcpA proteins of *A. actinomycetemcomitans* and *P. aeruginosa*. Both proteins RSP1087 and RSC0655 exhibit greater amino acid sequence identity with the RcpA protein of *P. aeruginosa* (40% and 37% sequence identity, respectively) compared with that of *A. actinomycetemcomitans* (28% and 33% sequence identity, respectively).

Proteins RSP1087 (454 amino acid residues, 47.2 kDa) and RSC0655 (634 amino acid residues, 64.8 kDa) both contain four transmembrane domains and are predicted to be localized to the outer membrane. The first 28 amino acids of protein RSP1087 possess characteristics of a signal peptide, with the cleavage site predicted to be between Ala_{27} and

Gln_{28} in the sequence $_{23}$PAAHA↓QGMPA$_{32}$. Cleavage at this site would yield a mature protein of 44.6 kDa. Likewise, the first 39 amino acids of the protein RSC0655 possess characteristics of a signal peptide, with the cleavage site predicted to be between Ala_{38} and Gln_{39} in the sequence $_{34}$GTAHA↓QAGVE$_{43}$, to yield a mature protein of 60.8 kDa.

Notably, both proteins RSP1087 and RSC0655 possess a secretion protein motif (GGX$_{12}$VP[L/F]LXXIPXIGXL[F/L]), located near the C-terminus of the proteins (Fig. 2.7a). This motif resembles that of the bacterial T2S system protein D (GspD). Protein D is involved in the general (type II) secretion pathway within Gram-negative bacteria and, being located in the outer membrane, is thought to be involved in transporting exoproteins from the periplasm, across the outer membrane, to the extracellular environment (Hobbs and Mattick, 1993). The notion that these *R. solanacearum* proteins may function as a secretin is furthermore supported by BLAST-P analysis, which indicated that the proteins share significant sequence homology to PilQ and members of the GspD/PulD family of outer membrane proteins. These proteins, also referred to as secretins, are involved in pilus biogenesis and extracellular secretion, respectively (Russel, 1998; Nouwen *et al.*, 1999). They are believed to function as a gated channel in the outer membrane through which protein substrates are secreted (Russel *et al.*, 1997; Guilvout *et al.*, 1999; Nouwen *et al.*, 1999). Within this family of outer membrane secretin proteins the RcpA/CpaC proteins of Tad systems, including the *R. solanacearum* proteins RSP1087 and RSC0655, formed a distinct clade that is most closely related to the GspD/PulD family of secretion proteins (Fig. 2.7b).

Homologues to RcpC

The RcpC protein appears to be unique to Flp biogenesis and secretion systems (Tomich *et al.*, 2007). In the case of *A. actinomycetemcomitans*, the RcpC protein is thought to be responsible for glycosylating the Flp pili (Tomich *et al.*, 2006) and/or to facilitate extrusion of the glycosylated Flp pili through the secretin ring (Tomich *et al.*, 2007). It has also been suggested that the RcpC protein of *P. aeruginosa* may be responsible for modifying the Flp pilin, thus promoting Flp-dependent adhesion to eukaryotic cells (Bernard *et al.*, 2009).

Homology searches indicated that two proteins encoded by the *tad* loci of *R. solanacearum* display significant sequence identity with the RcpC protein of *P. aeruginosa* only. The megaplasmid-encoded protein RSP1089 and the chromosome-encoded protein RSC0656 displayed 37% and 29% amino acid sequence identity, respectively, with the RcpC protein.

Fig. 2.7a Alignment of the putative secretin proteins of *R. solanacearum* with several members of the PilQ and GspD/PulD family of outer membrane proteins. Only the regions of each protein, containing the secretion protein motif (underlined), is shown. The putative secretin protein encoded by the megaplasmid *tad* locus of *R. solanacearum* is indicated by a circle, whilst that encoded by the chromosomal *tad* locus is indicated by a square.

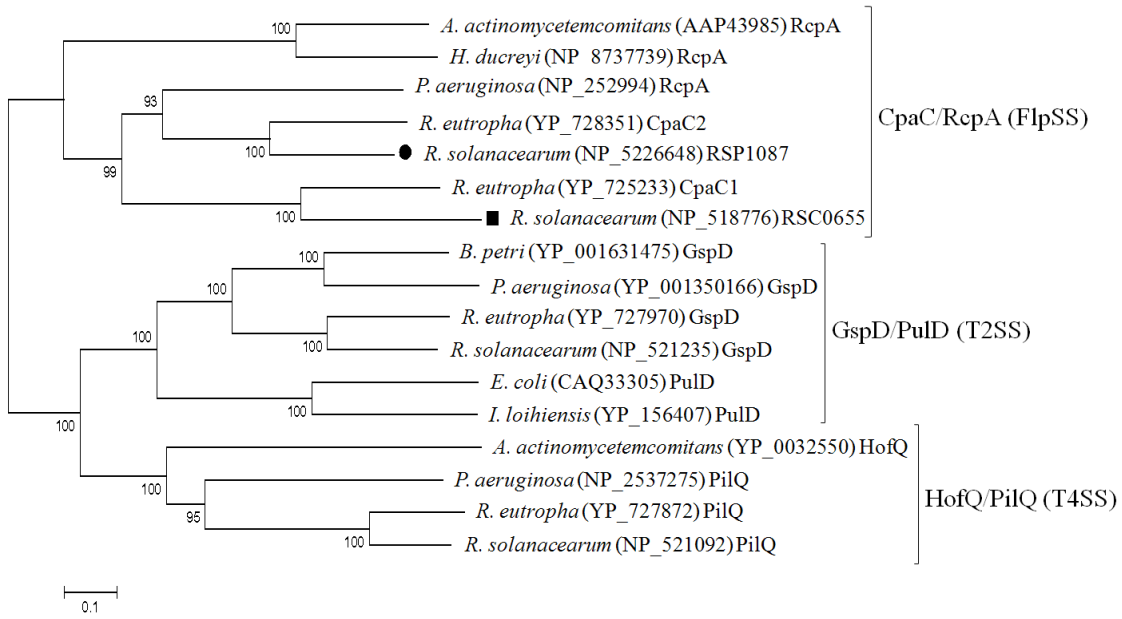


Fig. 2.7b Phylogeny of secretin proteins. The tree was generated with Neighbor-Joining distance analysis, and bootstrap support values are indicated at the internodes. The putative secretin protein encoded by the megaplasmid *tad* locus of *R. solanacearum* is indicated by a circle, whilst that encoded by the chromosomal *tad* locus is indicated by a square. GenBank sequence accession numbers are indicated in brackets. Abbreviations: FlpSS, Flp pilus secretion system; T2SS, Type II secretion system; T4SS, Type IV secretion system.

Proteins RSP1089 (208 amino acids, 22.6 kDa) and RSC0656 (288 amino acids, 30.4 kDa) are both predicted to be localized to the outer membrane and they each contain two transmembrane domains. Both these proteins also contain an apparent signal peptide sequence. The first 25 amino acids of RSP1089 possess characteristics of a signal peptide, with the cleavage site predicted to be between Ala_{24} and Trp_{25} in the sequence $_{20}$LGVVA↓WKNAH$_{29}$, whereas the first 30 amino acids of RSC0656 possess characteristics of a signal peptide, with the cleavage site predicted to be between Ile_{29} and Gln_{30} in the sequence $_{25}$SRWLI↓QQGNS$_{34}$. Cleavage at these sites would yield mature proteins of 20.2 kDa and 27.2 kDa, respectively. Notably, both these proteins were identified as belonging to the SAF superfamily, which comprises proteins with β-clip structural motifs. In agreement with this prediction, it has been reported that the RcpC proteins, including those of *A. actinomycetemcomitans*, *H. ducreyi* and *P. aeruginosa*, contain two β-clip motifs (Iyer and Aravind, 2004). It was furthermore proposed that these motifs may function by binding carbohydrate moieties of peptidoglycan to assemble structures like the Flp pili.

Homologues to TadD

In contrast to the TadD protein of *P. aeruginosa*, some functional studies have been performed on the homologous protein of *A. actinomycetemcomitans*. The TadD protein of *A. actinomycetemcomitans* is reported to be a lipoprotein and its expression is required for multimerization and localization of the RcpA secretin to the outer membrane (Clock *et al.*, 2008). It has also been suggested that TadD may influence the assembly, transport and/or function of the individual outer membrane Rcp proteins (Tomich *et al.*, 2007).

During the assembly and annotation of the *R. solanacearum* GMI1000 genome, proteins RSP1082 and RSC0654, encoded by the megaplasmid and chromosomal *tad* loci, respectively, had been annotated as lipoproteins (Salanabout *et al.*, 2002). Homology searches revealed that protein RSC0654 does not display homology with any known protein. In contrast, protein RSP1082 displays significant amino acid sequence identity (28%) with the TadD protein of *P. aeruginosa*.

Analysis of proteins RSP1082 (305 amino acid residues, 32.6 kDa) and RSC0654 (102 amino acid residues, 10.8 kDa), which are predicted to be localized to the outer membrane, indicated that they both contain a single transmembrane domain and a predicted site for N-terminal

cleavage by signal peptidase II. In prokaryotes, signal peptidase II is responsible for cleaving membrane lipoproteins and cleaves upstream of a cysteine (C) residue to which a glyceride-fatty acid lipid is then attached (Hayashi and Wu, 1990; Paetzel *et al.*, 2000). Such lipoprotein modification provides a versatile mechanism by which proteins may be anchored within the cell envelope (Sutcliffe and Russell, 1995). The signal peptide cleavage sites are predicted to be between Gly_{26} and Cys_{27} in the sequence $_{22}$ALLAG↓CGSLS$_{31}$ of protein RSP1082, and between Gly_{22} and Cys_{23} in the sequence $_{18}$ALLAG↓CMTTT$_{27}$ of protein RSC0654. Cleavage at these sites would yield mature proteins of 30 kDa and 8.6 kDa, respectively. A possible outer membrane localization of these proteins is furthermore supported by the absence of aspartate residues at position +2 of the mature proteins. It has been reported that the presence of these residues in mature lipoproteins function to retain them in the inner membrane, whilst the presence of other residues allows lipoprotein targeting to the outer membrane (Yamaguchi *et al.*, 1988).

In contrast to protein RSC0654, the amino acid sequence of protein RSP1082 contains two tetratricopeptide repeat (TPR) domains, spanning amino acid residues 138-171 and 172-205. Not only do TPR domains facilitate protein-protein interactions (Bayan *et al.*, 2006; Clock *et al*., 2008), but they are also common among a class of outer membrane lipoproteins, called docking proteins. These docking lipoproteins have been implicated in the assembly of secretin oligomers, in outer membrane insertion of secretins and in general stabilization of assembled secretin complexes (Bayan *et al.*, 2006). Interestingly, these results appear to suggest that protein RSP1082, but not RSC0654 encoded by the chromosomal *tad* locus, may be a pilotin with functions analogous to the TadD protein of *A. actinomycetemcomitans, i.e.* to ensure proper assembly and/or function of the secretin complex (Tomich *et al*., 2007).

2.3.3.6 Proteins encoded by the *tad* loci of *R. solanacearum* to which a function could not be assigned unambiguously

Protein RSP1088

Protein RSP1088, annotated as a hypothetical protein, is encoded by the *tad* locus in the megaplasmid genome of *R. solanacearum*. BLAST-P searches indicated that the protein displays significant amino acid sequence identity (66%) with the uncharacterized CpaB protein of *Ralstonia picketti* (GenBank accession no. ACS65600). The CpaB proteins are considered to be homologues of the RcpC protein and, in the case of *C. crescentus*, has been

reported to be involved in pili biogenesis, although its exact function is not known (Skerker and Shapiro, 2000). However, compared to the RcpC homologues identified earlier in these analyses (proteins RSP1089 and RSC0656), protein RSP1088 is *ca.* half the size (105 amino acid residues, 10.6 kDa) and lacks an apparent N-terminal signal peptide sequence. Moreover, no putative or conserved domains, *e.g.* β-clip motifs associated with RcpC proteins, could be identified in protein RSP1088. Further research would therefore be required to elucidate the possible function of protein RSP1088.

Protein RSP1081

Protein RSP1081, annotated as a signal peptide protein, is encoded by the *tad* locus present in the megaplasmid genome of *R. solanacearum*. BLAST-P searches indicated that protein RSP1081 exhibits 33% amino acid sequence identity with PA4298, an uncharacterized protein encoded only by the *tad* locus present in *P. aeruginosa* (de Bentzmann *et al.*, 2006). These proteins have a number of features in common; they are of similar size (RSP1081: 109 amino acid residues, 10.5 kDa and PA4298: 94 amino acid residues, 10.4 kDa), both are predicted to contain a single transmembrane domain and they each contain a putative N-terminal signal peptide sequence. However, clarification regarding their possible function in Flp pili biogenesis and secretion awaits further research.

Protein RSP1079

Protein RSP1079, annotated as a sigma 54 (σ^{54})-interacting transcriptional regulator protein, is encoded by the *tad* locus in the megaplasmid genome of *R. solanacearum*. Protein RSP1079 is comprised of 467 amino acid residues with an estimated molecular mass of 51.7 kDa. The RSP1084 protein lacks an apparent signal peptide sequence and is predicted to be localized to the cytoplasm, although it may be associated with the inner membrane via a single transmembrane domain. BLAST-P homology searches indicated that the protein belongs to the NtrC family of response regulators. In addition to a N-terminal receiver domain that is the site of phosphorylation and a C-terminal DNA binding domain, they also possess an additional central domain that is responsible for ATP hydrolysis and interaction with σ^{54} (Buck *et al.*, 2000). Analysis of the protein sequence of RSP1079 indicated that it contains a σ^{54} interaction domain and is predicted to have NTPase activity through two ATP-binding signature sequences ($_{161}$VMLYGETGAGKERI$_{174}$ and $_{224}$GYFEQANGGTLFLDEL$_{239}$). Moreover, protein RSP1079 also contains a helix-turn-helix (HTH) DNA-binding motif in its

C-terminus that resembles that of the Factor for Inversion Stimulation (FIS) protein. The latter is a regulator of different bacterial functions and has been shown to play a role in the regulation of virulence factors in both *Salmonella typhimurium* and *Escherichia coli* (Wilson *et al.*, 2001; Goldberg *et al.*, 2001). Notably, protein RSP1079 displays 38% amino acid sequence identity with the PilR protein of *P. aeruginosa*, which is an activator of T4ap expression (Mattick *et al.*, 1996; Darzins and Russel, 1997). Based on the presence of domains characteristic of prokaryotic regulatory proteins and its homology to PilR, it is tempting to speculate that protein RSP1079 may be involved in regulating expression of the megaplasmid *tad* locus of *R. solanacearum*. In this regard, it is particularly interesting to note that *tad* gene expression in *P. aeruginosa* is positively controlled by a PprB response regulator, which is present within the *tad* locus (Bernard *et al.*, 2009).

2.4 DISCUSSION

Secretion of proteins such as extracellular proteases, pili and toxins can provide selective advantages to bacteria in various environmental niches, and many of the secreted proteins are important colonization and virulence factors released by pathogenic bacteria. Of these, pili, found on a wide variety of Gram-negative bacteria, play an important role in adherence of pathogenic bacteria to their host (Hahn, 1997; Kang *et al.*, 1997), biofilm formation (O'Toole and Kolter, 1998), twitching motility (Wall and Kaiser, 1999; Craig *et al.*, 2004), conjugative DNA transfer (Krause *et al.*, 2000; Christie, 2001) and bacteriophage infection (Mattick *et al.*, 1996; Skerker and Shapiro, 2000). In addition to the well characterized T4ap, studies on several human bacterial pathogens have revealed the presence of T4bp, termed Flp pili, that mediate adherence to surfaces, formation of microcolonies and biofilms (Kachlany *et al.*, 2000; Nika *et al.*, 2002; de Bentzmann *et al.*, 2006), and contribute to the virulence of some bacterial species (Fuller *et al.*, 2000; Schreiner *et al.*, 2003; Spinola *et al.*, 2003).

In this part of the study, two distinct *tad* gene clusters were identified in the megaplasmid (RSP1079-1092) and chromosome (RSC0648-0661) of *R. solanacearum*, respectively, that encode predicted proteins that are similar to those involved in the biogenesis and secretion of Flp pili in both *A. actinomycetemcomitans* and *P. aeruginosa* (Tables 2.2 and 2.3). 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

Table 2.2 Characteristics of the Tad system proteins encoded by the *R. solanacearum* megaplasmid *tad* locus, listed together with homologous proteins of *A. actinomycetemcomitans* and *P. aeruginosa*

Protein name[a]	RSP number[a]	Homologue[b]	Percent identity[bc]	Percent similarlity[bc]	Protein length (aa)[d]	Protein weight (kDa)[d]	Predicted protein location and function [e]
Flp	**1092**				**58**	**5.9**	**IM/Secreted ; Pilin**
		Flp2 / *A. actinomycetemcomitans*	(9/17) 52	(13/17) 76	75	8.1	
		Flp1 / *A. actinomycetemcomitans*	(15/29) 51	(20/29) 68	76	8.3	
		Flp / *P. aeruginosa*	(23/54) 42	(32/54) 59	72	7.3	
		RSC0660 / *R. solanacearum*	**(22/53) 41**	**(32/53) 60**	**53**	**5.7**	
		RSC0661 / *R. solanacearum*	**(21/53) 39**	**(31/53) 58**	**53**	**5.8**	
		RSC0658 / *R. solanacearum*	**(16/53) 30**	**(27/53) 50**	**53**	**5.7**	
		RSC0659 / *R. solanacearum*	**NSH**		**45**	**4.8**	
CpaA2	**1091**				**168**	**17.8**	**IM ; Prepilin peptidase**
		FppA / *P. aeruginosa*	(27/74) 36	(38/74) 51	160	17.5	
		TadV / *A. actinomycetemcomitans*	(23/65) 33	(36/65) 55	142	15.8	
		RSC0657 / *R. solanacearum*	**(48/152) 31**	**(64/152) 42**	**172**	**17.3**	
TadG2	**1090**				**163**	**17.0**	**IM ; Pseudopilin**
		RSC0649 / *R. solanacearum*	**(23/55) 41**	**(35/55) 63**	144	15.2	
		TadF / *P. aeruginosa*	(17/55) 30	(27/55) 49	168	18.1	
		TadF / *A. actinomycetemcomitans*	NSH		200	23.0	
		TadE / *A. actinomycetemcomitans*	NSH		191	21.7	
CpaB2	**1089**				**208**	**22.6**	**OM ; Unknown**
		RcpC / *P. aeruginosa*	(56/151) 37	(78/151) 51	303	31.8	
		RSC0656 / *R. solanacearum*	**(47/162) 29**	**(83/162) 51**	**288**	**30.3**	
		RSP1088 / *R. solanacearum*	**NSH**		**105**	**10.6**	
		RcpC / *A. actinomycetemcomitans*	NSH		274	30.4	
Hypothetical	**1088**				**105**	**10.6**	**Cyt ; Unknown**
		NSH to known proteins					
CpaC2	**1087**				**454**	**47.2**	**OM ; Secretin**
		RcpA / *P. aeruginosa*	(156/388) 40	(225/388) 57	416	44.3	
		RSC0655 / *R. solanacearum*	**(125/432) 28**	**(213/432) 49**	**634**	**64.8**	
		RcpA / *A. actinomycetemcomitans*	(117/441) 28	(198/441) 44	460	50.1	
CpaE2	**1086**				**439**	**47.5**	**Cyt ; Localization?**
		TadZ / *P. aeruginosa*	(95/330) 28	(163/330) 49	394	42.4	
		RSC0653 / *R. solanacearum*	**(63/286) 22**	**(121/286) 42**	**397**	**43.0**	
		TadZ / *A. actinomycetemcomitans*	NSH		317	41.6	
CpaF2	**1085**				**450**	**49.4**	**IM/Cyt ; ATPase**
		TadA / *P. aeruginosa*	(193/391) 49	(269/391) 68	421	46.8	
		TadA / *A. actinomycetemcomitans*	(167/345) 48	(242/345) 70	426	47.1	
		RSC0652 / *R. solanacearum*	**(182/386) 47**	**(268/386) 69**	**453**	**50.6**	
TadB2	**1084**				**308**	**33.9**	**IM ; Scaffold?**
		TadB / *P. aeruginosa*	(60/177) 33	(103/177) 58	294	32.4	
		TadB / *A. actinomycetemcomitans*	(38/144) 26	(65/144) 45	295	34.4	
		RSC0651 / *R. solanacearum*	**(53/210) 25**	**(99/210) 47**	**325**	**36.0**	
TadC2	**1083**				**326**	**35.9**	**IM ; Scaffold?**
		TadC / *P. aeruginosa*	(75/234) 32	(135/234) 57	303	33.7	
		RSC0650 / *R. solanacearum*	**(65/253) 25**	**(119/253) 47**	**315**	**34.9**	
		TadC / *A. actinomycetemcomitans*	(45/201) 22	(101/201) 50	288	32.2	
TadD2	**1082**				**305**	**32.6**	**OM ; Pilotin**
		TadD / *P. aeruginosa*	(63/221) 28	(98/221) 44	245	26.6	
		RSC0654 / *R. solanacearum*	**NSH**		**102**	**10.8**	
		TadD / *A. actinomycetemcomitans*	NSH		253	28.5	
Hypothetical	**1081**				**109**	**10.5**	**IM ; Unknown**
		PA4298 / *P. aeruginosa*	(32/95) 33	(44/95) 46	94	10.4	
Hypothetical	**1080**				**540**	**49.5**	**IM ; Unknown**
		TadG / *P. aeruginosa*	(130/442) 30	(208/442) 47	556	56.4	
		RSC0648 / *R. solanacearum*	**NSH**		**347**	**35.4**	
Hypothetical	**1079**				**467**	**51.7**	**IM ; Unknown**
		PilR / *P. aeruginosa*	(133/353) 38	(187/353) 53	452	50.6	

[a] *R. solanacearum* megaplasmid Tad system protein names and RSP numbers, as given during the genome annotation (Salanabout *et al.*, 2002).
[b] Homologues were identified using BLAST-P. *R. solanacearum* proteins are indicated in bold.
[bc] The percentage identity and similarity was calculated using BLAST-P. Proteins with no significant homology (NSH) are shown for comparison purpose.
[d] Length of predicted proteins and molecular mass were calculated for full-length proteins.
[e] Predicted localization of each *R. solanacearum* protein (cytoplasmic (Cyt); inner membrane (IM); periplasmic; outer membrane (OM); or secreted) was obtained using PSORT, SMART, SIGNALP and TMPRED.

Table 2.3 Characteristics of the Tad system proteins encoded by the *R. solanacearum* chromosomal *tad* locus, listed together with homologous proteins of *A. actinomycetemcomitans* and *P. aeruginosa*

Protein name[a]	RSC number[a]	Homologue[b]	Percent identity[bc]	Percent similarlity[bc]	Protein length (aa)[d]	Protein weight (kDa)[d]	Predicted protein location and/or function[e]
Flp4	**0661**				**53**	**5.7**	**IM/Secreted ; Pilin**
		RSC0660 / *R. solanacearum*	**(48/53) 90**	**(51/53) 96**	**53**	**5.7**	
		RSC0659 / *R. solanacearum*	**(26/45) 57**	**(33/45) 73**	**45**	**4.8**	
		RSC0658 / *R. solanacearum*	**(28/53) 52**	**(37/53) 69**	**53**	**5.7**	
		RSP1092 / *R. solanacearum*	**(21/53) 39**	**(31/53) 58**	**58**	**5.9**	
		Flp / *P. aeruginosa*	(10/37) 27	(22/37) 59	72	7.3	
		Flp1 / *A. actinomycetemcomitans*	NSH		76	8.3	
		Flp2 / *A. actinomycetemcomitans*	NSH		75	8.1	
Flp3	**0660**				**53**	**5.7**	**IM/Secreted ; Pilin**
		RSC0661 / *R. solanacearum*	**(48/53) 90**	**(51/53) 96**	**53**	**5.8**	
		RSC0659 / *R. solanacearum*	**(26/45) 54**	**(33/45) 73**	**45**	**4.8**	
		RSC0658 / *R. solanacearum*	**(29/53) 54**	**(38/53) 71**	**53**	**5.7**	
		RSP1092/ *R. solanacearum*	**(22/53) 41**	**(32/53) 60**	**58**	**5.9**	
		Flp / *P. aeruginosa*	(9/38) 23	(23/38) 60	72	7.3	
		Flp1 / *A. actinomycetemcomitans*	NSH		76	8.3	
		Flp2 / *A. actinomycetemcomitans*	NSH		75	8.1	
Flp2	**0659**				**45**	**4.8**	**IM/Secreted ; Pilin**
		RSC0661 / *R. solanacearum*	**(26/45) 57**	**(33/45) 73**	**53**	**5.8**	
		RSC0660 / *R. solanacearum*	**(26/45) 54**	**(33/45) 73**	**53**	**5.7**	
		RSC0658 / *R. solanacearum*	**(20/45) 44**	**(27/45) 60**	**53**	**5.7**	
		RSP1092/ *R. solanacearum*	**NSH**		**58**	**5.9**	
		Flp1 / *A. actinomycetemcomitans*	NSH		76	8.3	
		Flp2 / *A. actinomycetemcomitans*	NSH		75	8.1	
		Flp / *P. aeruginosa*	NSH		72	7.3	
Flp1	**0658**				**53**	**5.7**	**IM/Secreted ; Pilin**
		RSC0660 / *R. solanacearum*	(29/53) 54	(38/53) 71	53	5.7	
		RSC0661 / *R. solanacearum*	(28/53) 52	(37/53) 69	53	5.8	
		RSC0659 / *R. solanacearum*	(20/45) 44	(27/45) 60	44	4.8	
		Flp / *P. aeruginosa*	(10/29) 34	(21/29) 72	72	7.3	
		RSP1092/ *R. solanacearum*	**(16/53) 30**	**(27/53) 50**	**58**	**5.9**	
		Flp1 / *A. actinomycetemcomitans*	(12/39) 30	(23/39) 58	76	8.3	
		Flp2 / *A. actinomycetemcomitans*	NSH		75	8.1	
CpaA1	**0657**				**172**	**17.3**	**IM ; Prepilin peptidase**
		TadV / *A. actinomycetemcomitans*	(24/70) 33	(32/70) 45	142	15.8	
		FppA / *P. aeruginosa*	(37/144) 25	(60/144) 41	160	17.5	
CpaB1	**0656**				**288**	**30.4**	**OM ; Unknown**
		RcpC / *P. aeruginosa*	(38/129) 29	(62/129) 48	303	31.8	
		RcpC / *A. actinomycetemcomitans*	NSH				
CpaC1	**0655**				**634**	**64.8**	**OM ; Secretin**
		RcpA / *P. aeruginosa*	(101/266) 37	(141/266) 53	416	44.3	
		RcpA / *A. actinomycetemcomitans*	(58/165) 33	(94/165) 56	460	50.1	
Hypothetical	**0654**				**102**	**10.8**	**OM ; Unknown**
		NSH to known proteins					
CpaE1	**0653**				**397**	**43.0**	**Cyt ; Localization?**
		TadZ / *P. aeruginosa*	(68/297) 22	(118/297) 39	394	42.4	
		TadZ / *A. actinomycetemcomitans*	(41/205) 20	(78/205) 38	317	41.6	
CpaF1	**0652**				**453**	**50.6**	**IM/Cyt ; ATPase**
		TadA / *P. aeruginosa*	(195/381) 51	(270/381) 70	421	46.8	
		TadA / *A. actinomycetemcomitans*	(185/396) 46	(280/396) 70	426	47.1	
TadB1	**0651**				**325**	**36.0**	**IM ; Scaffold?**
		TadB / *P. aeruginosa*	(43/155) 27	(80/155) 51	294	32.4	
		RSP1084 / *R. solanacearum*	**(53/210) 25**	**(99/210) 47**	**308**	**33.9**	
		TadB / *A. actinomycetemcomitans*	(43/192) 22	(82/192) 42	295	34.4	
TadC1	**0650**				**315**	**34.8**	**IM ; Scaffold?**
		TadC / *P. aeruginosa*	(77/288) 26	(125/288) 43	303	33.7	
		TadC / *A. actinomycetemcomitans*	(47/200) 23	(103/200) 51	288	32.2	
TadG1	**0649**				**144**	**15.2**	**IM ; Pseudopilin**
		TadF / *P. aeruginosa*	(12/32) 37	(19/32) 59	168	18.1	
		TadE / *A. actinomycetemcomitans*	(13/42) 30	(22/42) 52	191	21.7	
		TadF / *A. actinomycetemcomitans*	NSH		200	23.0	
Hypothetical	**0648**				**347**	**35.4**	**IM ; Unknown**
		TadG / *P. aeruginosa*	(20/50) 40	(27/50) 54	556	56.4	

[a] *R. solanacearum* megaplasmid Tad system protein names and RSC numbers, as given during the genome annotation (Salanabout *et al*., 2002).
[b] Homologues were identified using BLAST-P. *R. solanacearum* proteins are indicated in bold.
[bc] The percentage identity and similarity was calculated using BLAST-P. Proteins with no significant homology (NSH) are shown for comparison purpose.
[d] Length of predicted proteins and molecular mass were calculated for full-length proteins.
[e] Predicted localization of each *R. solanacearum* protein (cytoplasmic (Cyt); inner membrane (IM); periplasmic; outer membrane (OM); or secreted) was obtained using PSORT, SMART, SIGNALP and TMPRED.

(recombination) and horizontal gene transfer between distant bacterial relatives (Planet *et al.*, 2001; 2003). Consequently, it has been proposed that the *tad* locus is a mobile genome island, termed the "widespread colonization island" (WCI), in order to reflect its requirement for the colonization of diverse environmental niches (Planet *et al.*, 2003). Given that horizontal gene transfer may play an important role in the evolution of the *tad* locus, the G+C content of the respective *R. solanacearum tad* loci was examined and found to be similar to the average G+C content of the genome (66.97%) (Salanoubat *et al.*, 2002). Although such sequence composition analyses is considered to be inaccurate indicators of horizontally transferred genes (Eisen *et al.*, 2000; Koski *et al.*, 2000), the results do not exclude the possibility that the *tad* gene cluster may have been acquired either from a bacterial species with a similar nucleotide composition to that of *R. solanacearum* or through an ancient horizontal gene transfer event. This is not unlikely as *R. solanacearum* has a propensity to acquire and recombine exogenous DNA through natural transformation (Bertolla *et al.*, 1997). Nevertheless, determination of ancestry of the *R. solanacearum tad* gene cluster, as well as establishment of a relationship between the megaplasmid and chromosome *tad* genes in terms of paralogy must await a more comprehensive analysis.

As indicated under Results, homologues to most of the Tad system proteins are encoded by the *tad* gene clusters present in the megaplasmid and chromosome of *R. solanacearum*. In the case of *R. solanacearum* the respective *tad* loci encode one and four Flp putative proteins, respectively. Flp1 pilin is the major structural component of the *A. actinomycetemcomitans* Flp pili, and the mature pilin is a product of a proteolytically modified pre-Flp1 protein, encoded by *flp-1* (Kachlany *et al.*, 2001; Tomich *et al.*, 2006). Despite containing two *flp* alleles, it has been shown that *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). Although the predicted singular Flp protein (RSP1092) encoded by the *R. solanacearum* megaplasmid *tad* locus shares features typical of Flp proteins (Kachlany *et al.*, 2001; Perez *et al.*, 2006), the predicted four Flp proteins (RSC0658-0661) encoded by the chromosomal *tad* locus show differences in these features. The Flp motif is absent in protein RSC0659 and all of the putative proteins are missing a conserved Phe residue in their C-terminus that is present in most predicted Flp proteins (Fig. 2.3a). Why the *R. solanacearum* chromosomal *tad* locus has the genetic information to produce three similar Flp-like proteins (RSC0658, RSC0660 and RSC0661) is not clear. It is nevertheless interesting to note that these three Flp putative proteins have identical residues at equivalent positions, most notably the sequence of the

putative prepilin peptidase site (G/X_4EY) (Kachlany *et al.*, 2001; Tomich *et al.*, 2006), suggesting conservative maintenance of key functional amino acids by natural selection. Thus, whether one or more of the putative *flp* genes of the chromosomal *tad* locus may represent pseudogenes that have become dispensable for Flp pilin biogenesis, as in the case of *flp-2* of *A. actinomycetemcomitans*, or might still have a function remains to be determined.

The *R. solanacearum tad* loci are predicted to each encode a single pilus-like protein (RSP1090 and RSC0649) that displays homology with the TadE and TadF pseudopilin proteins. Pseudopilins are components of T2S systems in different Gram-negative bacteria, including the T2S system of *Klebsiella oxytoca* (Pugsley and Dupuy, 1992), the Xps system of *Xanthomonas campestris* (Hu *et al.*, 2002) and the Xcp system of *P. aeruginosa* (Lu *et al.*, 1997; Bleves *et al.*, 1998). Based on their ability to form a periplasmic pilus-like structure, it has been suggested that the pseudopilins may function in extruding the substrate through intermittent rounds of polymerization and depolymerization (Hobbs and Mattick, 1993; Sandkvist, 2001). It is, however, unclear what roles the pilus-like proteins may play in bacterial pilus biogenesis, and especially in Flp pili biogenesis. Reports in this regard have been conflicting, indicating that they are indispensible for Flp biogenesis in *A. actinomycetemcomitans* (Tomich *et al.*, 2006) but not required for Flp biogenesis in *P. aeruginosa* (Bernard *et al.*, 2009). These results may thus suggest that despite the presence of homologous Tad proteins, Tad systems in different bacteria may have slightly different modes of operation.

The pilin and pseudopilin proteins of Flp biogenesis and secretion systems are cleaved by prepilin peptidases, namely TadV of *A. actinomycetemcomitans* (Tomich *et al.*, 2006) and FppA of *P. aeruginosa* (de Bentzmann *et al.*, 2006). The predicted gene products RSP1091 and RSC0656, encoded by the *tad* loci in the *R. solanacearum* megaplasmid and chromosome, respectively, have homology to TadV/FppA, and to type IV pilus (T4P) and T2S system prepilin peptidases. The *R. solanacearum* proteins are predicted to lack a cleavable signal sequence and to have four transmembrane domains, which are features consistent with a possible function as a prepilin peptidase (Strom *et al.*, 1993; La Pointe *et al.*, 2000; Akahane *et al.*, 2005). In addition, proteins RSP1091 and RSC0656 contain two conserved aspartic acid residues that have been shown to be critical for the catalytic function of the *A. actinomycetemcomitans* TadV (Tomich *et al.*, 2006) and the *P. aeruginosa* FppA (de

Bentzmann *et al.*, 2006) prepilin peptidases. It is therefore likely that the predicted proteins RSP1091 and RSC0656 may function in the maturation of the *R. solanacearum* Flp pili.

The predicted amino acid sequence of the *R. solanacearum* proteins RSP1085 and RSC0652 showed significant homology with the TadA proteins of *A. actinomycetemcomitans* and *P. aeruginosa*, as well as to members of the TrbB/VirB11 family of secretion NTPases. The proteins are predicted to be localized to the cytoplasm and to be associated with the inner membrane. This is typical of other secretion NTPases such as the PulE protein of the T2S system for pullulanase secretion (Possot and Pugsley, 1994; 1997), as well as the T4S system NTPase VirB11, encoded by Ti plasmids (Rashkova *et al.*, 1997). The *R. solanacearum* proteins contain two conserved motifs for nucleotide binding and hydrolysis, commonly found in NTPases: Walker A and B boxes (Walker *et al.*, 1982) and an Asp box (Sandkvsit *et al.*, 1995). A His box of unknown function and conserved in T4S system NTPases is also present (Whitchurch *et al.*, 1991; Possot and Pugsley, 1994). Mutations introduced into the *tadA* genes of *A. actinomycetemcomitans* (Kachlany *et al.*, 2000; Bhattacharjee *et al.*, 2001), *P. aeruginosa* (de Bentzmann *et al.*, 2006) and *H. ducreyi* (Nika *et al.*, 2002) were reported to result in bacterial strains lacking Flp pili. The NTPases of T4P, T4S and T2S systems have been shown to interact with other components of the secretion apparatus that are embedded in the inner membrane (Grahn *et al.*, 2000; Krause *et al.*, 2000; Rashkova *et al.*, 2000). The most likely candidates for interaction with the *R. solanacearum* TadA putative proteins are homologues of the TadB (RSP1084 and RSC0651) and TadC (RSP1083 and RSC0650) proteins. These four *R. solanacearum* putative proteins are predicted to be inner membrane proteins and, in homology searches, showed similarity to PilC-like proteins, which are integral inner membrane proteins of the T2S system (Peabody *et al.*, 2003). Thus, it is likely that these proteins may be required for stability of other proteins of the Tad systems, potentially as an inner membrane scaffold. Alternatively, these proteins may act as molecular pistons that channel energy generated by TadA into Flp polymerization, as had been suggested for *A. actinomycetemcomitans* (Tomich *et al.*, 2007).

Sequence predictions indicated that three putative proteins of each of the respective *R. solanacearum tad* loci may localize to the bacterial outer membrane. These include a homologue of the RcpA protein (RSP1087 and RSC0655), which are like RcpA of *A. actinomycetemcomitans* and *P. aeruginosa*, members of the GspD/PulD family of outer membrane proteins. Moreover, all of these proteins contain a C-terminal motif that is

conserved among known secretin proteins, including PulD required for pullulanase secretion and PilQ, an assembly protein of T4ap (Martin *et al.*, 1993; Drake and Koomey, 1995). Therefore, it is likely that the putative proteins RSP1087 and RSC0655 may each operate as a secretin through which Flp pili of the respective Tad secretion systems traverse the outer membrane. In contrast, predicted homologues of the outer membrane protein RcpC (RSP1089 and RSC0656) do not have a strongly predicted function. However, these *R. solanacearum* proteins do contain two β-clip motifs that have been suggested previously to facilitate assembly of Flp pili (Iyer and Aravind, 2004). An RcpC-dependent electrophoretic mobility shift of Flp pilin has been reported in *A. actinomycetemcomitans* (Inoue *et al.*, 2000; Tomich *et al.*, 2006) and *P. aeruginosa* (Bernard *et al.*, 2009), indicating that RcpC may be involved in post-translational modification of Flp pili. Interestingly, in *P. aeruginosa*, an *rcpC* mutation had no effect on adherence to an abiotic surface, but decreased adhesion to epithelial cells (Bernard *et al.*, 2009). These results could suggest that the RcpC protein is multifunctional, with independent roles in Flp assembly, modification and adhesion. Both *R. solanacearum* *tad* loci are predicted to encode lipoproteins that may localize to the bacterial outer membrane. However, only protein RSP1082, encoded by the megaplasmid *tad* locus, displays homology with the TadD lipoproteins of bacterial Tad systems. In contrast, the predicted lipoprotein encoded by the chromosomal *tad* locus (RSC0654) is truncated (being one third of the length of Tad homologues) and lacks the typical domains associated with TadD proteins. The protein sequence of RSP1082 contains, in addition to a predicted site for N-terminal cleavage by signal peptidase II, a TPR domain that is important for interaction during secretion complex assembly (Bayan *et al.*, 2006; Nudleman *et al.*, 2006). The TPR domain is also a common feature among docking proteins, a class of outer membrane lipoproteins, such as PilW of *Neisseria meningitides* and Tgl of *Myxococcus xanthus*, which have known roles in positioning secretin proteins at the outer membrane (Carbonnelle *et al.*, 2006; Nudleman *et al.*, 2006).

It has been proposed previously that the RcpA protein, together with the RcpC and TadD proteins, constitute an outer membrane complex unique to Tad machines (de Bentzmann *et al.*, 2006; Tomich *et al.*, 2007). The lack of a discernible *tadD* homologue in the chromosomal *tad* locus of *R. solanacearum* is therefore particularly interesting, especially since it was reported that RcpA could not be detected in a *tadD* mutant of *A. actinomycetemcomitans* (Clock *et al.*, 2008). It was shown that without TadD there is severe destabilization and near complete degradation of the RcpA protein. Thus, TadD appears to

play a crucial role in mediating the association of the RcpA subunits and acting as an assembly factor for the insertion of RcpA into the outer membrane. Notably, the abundance of RcpC in the *tadD* mutant was also reported to be reduced, albeit not as drastically as in the case of RcpA (Clock *et al.*, 2008). Based on the above, it is therefore tempting to speculate that the chromosomal *tad* locus of *R. solanacearum* may not be capable of assembling into a functional Flp secretion system. 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 a 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).

In silico analysis of the *R. solanacearum tad* loci also led to the identification of putative *tad* gene cluster promoters that resemble *E. coli* σ^{70} promoter sequences (Fig. 2.2). In this regard, it is interesting to note that a σ^{70} promoter upstream of the *flp-1* gene was necessary and sufficient for transcription of the *tad* locus of *A. actinomycetemcomitans* (Wang and Chen, 2005; Kram *et al.*, 2008). In contrast, the *P. aeruginosa tad* locus is transcribed as five independent transcriptional units and it was shown that gene expression was positively controlled by a response regulator, PrpB, which is transcribed independently within the *tad* gene cluster (Bernard *et al.*, 2009). Interestingly, in the case of *R. solanacearum*, the regions upstream of the *tad* gene clusters were also predicted to contain σ^{54} promoter sequences. This warrants further discussion, especially considering that protein RSP1079, encoded by the megaplasmid *tad* locus only, displays homology with PilR, an activator of pilin gene transcription (Mattick *et al.*, 1996).

Both protein RSP1079 and PilR belong to the NtrC family of response regulators (Stock *et al.*, 2000). These regulators bind to regions of DNA upstream of a σ^{54}-dependent promoter and, through ATP hydrolysis, convert the σ^{54} RNA polymerase into an open transcription complex, thereby allowing transcription to occur (Buck *et al.*, 2000; Zhang *et al.*, 2002). Expression of T4ap in *P. aeruginosa* and a variety of other Gram-negative bacteria has been reported to be regulated by σ^{54}, PilS (membrane-bound sensor kinase) and PilR (cytoplasmic response regulator) (Ishimoto and Lory, 1992; Hobbs *et al.*, 1993; Wu and Kaiser, 1997; Parker *et al.*, 2006; Li *et al.*, 2007). However, in this study, no homologue encoding a putative sensor for the RSP1079 response regulator could be identified within the megaplasmid *tad*

locus of *R. solanacearum*. The lack of a discernible sensor raises interesting questions regarding the activity of the putative RSP1079 regulator. In this regard, it is interesting to note that several bacterial species contain two-component response regulators with atypical activity. In *Helicobacter pylori*, the response regulator Hp166 has been shown to have activity in the absence of its cognate sensor kinase (Beier and Frank, 2000; Schär *et al.*, 2005). In *P. aeruginosa*, the AlgR response regulator is capable of activating a number of genes it regulates in the absence of its cognate sensor FimS (Ma *et al.*, 1998). Similarly, the *P. aeruginosa* AlgB response regulator has been shown to have activity in the absence of phosphorylation (Ma *et al.*, 1998; Leech *et al.*, 2008). In *Kingella kingae*, T4ap expression appears to be independent of the PilS sensor (Kehl-Fie *et al.*, 2009). Moreover, PrpB-dependent activation of the *tad* gene cluster in *P. aeruginosa* appears to be independent of its sensor kinase, PrpA (Bernard *et al.*, 2009). Cumulatively, these results indicate that expression of Flp pili by *R. solanacearum* may be complex and multilayered, and that *R. solanacearum* possibly combines aspects of regulation from both *A. actinomycetemcomitans* and *P. aeruginosa*.

In conclusion, based on the results of the *in silico* analyses performed, the putative Tad proteins encoded by the megaplasmid, and to a lesser extent those encoded by the chromosome, may be capable of forming a macromolecular structure for the assembly and secretion of Flp pili in *R. solanacearum*. By analogy with *A. actinomycetemcomitans* and *P. aeruginosa*, the *tad* genes of *R. solanacearum* may also be important for adherence and colonization of diverse environmental niches. Consequently, the elucidation of the specific roles of the Tad proteins could be important to understanding *R. solanacearum* colonization and pathogenesis. This knowledge may not only provide significant insights about the functions of Tad proteins in other phytopathogens, but may also lead to improved control strategies for this devastating phytopathogen.

CHAPTER THREE

A FLP PILUS BIOGENESIS AND SECRETION SYSTEM CONTRIBUTES TO ADHERENCE OF *Ralstonia solanacearum* TO ROOTS AND VIRULENCE *IN VIVO*

3.1 INTRODUCTION

Ralstonia solanacearum is a soil-dwelling Gram-negative bacterium that causes bacterial wilt disease in over 450 plant species (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). The bacterium enters the host through wounded roots or sites of secondary root emergence and once inside a host, it quickly spreads and multiplies (Schell, 2000; Vasse *et al.*, 2005). During plant infection, *R. solanacearum* colonizes the xylem vessels where the bacterium reaches populations of 10^{10} CFU/ml of xylem fluid (McGarvey *et al.*, 1999). *R. solanacearum* possesses various virulence factors that act quantitatively to cause disease. These include extracellular polysaccharide I (EPS I) (Denny and Baek, 1991; McGarvey *et al.*, 1999), cell wall-degrading enzymes (Gonzalez and Allen, 2003; Liu *et al.*, 2005), flagellar and twitching motility (Kao *et al.*, 1992; Tans-Kersten *et al.*, 2001; Liu *et al.*, 2001), and type III-secreted effectors (Cunnac *et al.*, 2004; Genin *et al.*, 2005). Most of the 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; Genin and Boucher, 2004). 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.

The ability of *R. solanacearum* to attach to the host is of special importance, since physical contact with the plant is required to induce expression of the type III secretion (T3S) system (Aldon *et al.*, 2000). This secretion system is responsible for delivering the majority of virulence factors into the host cells and thus contributes significantly to virulence (van Gijsegem *et al.*, 2000). It therefore follows that appendages that facilitate pathogen-host adherence may be important determinants in disease development. Based on the results of whole-genome sequence analysis, it has been reported that *R. solanacearum* may encode for an abundance of proteins that promote adherence of the bacterium (Salanoubat *et al.*, 2002; Genin and Boucher, 2004). In addition to flagella and type IVa pili (T4ap), which have been suggested to mediate movement to and inside the host tissues (Tans-Kersten *et al.*, 2001; Kang *et al.*, 2002), two distinct *tad*-related gene clusters have also been identified (Salanoubat *et al.*, 2002; Chapter 2). Notably, the *tad* gene cluster of *Aggregatibacter actinomycetemcomitans* was reported to be required for the biogenesis and secretion of a novel type IVb pilus (T4bp), designated Flp, which is required for tight non-specific

adherence of the bacterium to surfaces (Kachlany *et al*., 2000). Homologous gene clusters have since been identified in a wide variety of Gram-negative and Gram-positive bacteria (Planet *et al.*, 2003; Tomich *et al.*, 2007), and the encoded Flp pili have been implicated in processes such as adherence (Nika *et al.*, 2002; de Bentzmann *et al.*, 2006) and virulence (Fuller *et al*., 2000; Schreiner *et al*., 2003; Spinola *et al.*, 2003) of human pathogenic bacteria. However, the functional significance of the *tad* gene cluster in plant pathogens have not yet been determined or reported.

In the previous Chapter, an *in silico* approach was adopted to analyze the *tad* gene clusters present on the chromosome and megaplasmid of *R. solanacearum* GMI1000. Although the function of these genes in *R. solanacearum* may be deduced through *in silico* predictions and by comparative analysis of the respective genes, it is, however, only through the construction of mutations in *R. solanacearum* that their actual function in this bacterium can be elucidated. Various different strategies, including transposon mutagenesis and allelic exchange, have been described whereby mutant bacterial strains can be generated (Coelho *et al.*, 2000; Schweizer, 2008). Transposons, being mobile genetic elements, are capable of inserting themselves randomly into genes, thereby disrupting their function. Transposon mutagenesis therefore represents a potentially powerful approach towards identifying genes involved in a specific function, provided that a high-throughput screen is available (Reznikoff and Winterberg, 2008; Choi and Kim, 2009). Indeed, this approach was used to identify the *tad* gene cluster as being required for tight adherence of *A. actinomycetemcomitans* to surfaces (Kachlany *et al*., 2000). In contrast, allelic exchange involves using plasmids that are conditional for replication ("suicide plasmid") to deliver an *in vitro*-inactivated or -modified allele of the gene of interest into the genome (Skorupski and Taylor, 1996; Ortiz-Martín *et al.*, 2006). In such instances, a copy of a chromosomal gene of interest, which has been disrupted through the insertion of a suitable marker such as an antibiotic resistance gene, is cloned into a plasmid and then introduced into a recipient strain where the plasmid cannot replicate. Since the plasmid cannot replicate, selection for some property of the plasmid, such as the newly introduced antibiotic resistance marker, results in isolates that have integrated the cloned disrupted DNA fragment into the host genome via homology between the DNA fragment and the corresponding region of the recipient genome. Since mutations made by allelic exchange are targeted, it thus represents a powerful approach to elucidating gene function (Nika *et al.*, 2002; Wang and Chen, 2005).

Although flagella and T4ap have both been implicated in adherence of *R. solanacearum* to the host (Tans-Kersten *et al.*, 2001; Liu *et al.*, 2001), it is notable that a mutant of *R. solanacearum* deficient in T4a pili was reported to be capable of adhering to plant cells and roots, and was also capable of causing wilt disease in tomato plants (Kang *et al.*, 2002). Since fimbriae other than T4a pili may thus play a role in adherence of *R. solanacearum* to the host, and considering that the Flp pili of *A. actinomycetemcomitans*, *Haemophilus ducreyi* and *Pseudomonas aeruginosa* have been shown to play a role in adherence of these bacteria to different surfaces, the aim of this part of the investigation was to determine the importance of the Flp biogenesis and secretion system in the ability of *R. solanacearum* to adhere to and cause disease on potato plants. For this purpose, a *R. solanacearum* strain was generated in which the *cpaF2* (RSP1085) gene that is present in the megaplasmid *tad* locus was mutated. This gene encodes a putative NTPase that functions as the energizer for Flp pilus assembly (Bhattacharjee *et al.*, 2001), and is highly conserved in the *tad* loci of different bacteria (Planet *et al.*, 2001; Tomichi *et al*., 2007).

3.2 MATERIALS AND METHODS

3.2.1 Bacteria, plasmids and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 3.1. *R. solanacearum* strain NB336, a potato isolate, was obtained from Coen Bezuidenhout Seed Testing (Pretoria, South Africa). *R. solanacearum* strains were cultured at 30°C in casein hydrolysate-peptone-glucose (CPG) broth (0.1% [w/v] casein hydrolysate, 1% [w/v] peptone, 0.1% [w/v] yeast extract, 0.2% [w/v] glucose; pH 7.3-7.5) (Hendrick and Sequeira, 1984) or on 2, 3, 5-triphenyltetrazolium chloride (TZC) agar (CPG broth amended with 1.5% [w/v] bacteriological agar and 0.005% [v/v] TZC) (Kelman, 1954). *Escherichia coli* DH5α strains were cultured at 37°C in Luria-Bertani (LB) broth (1% [w/v] tryptone, 1% [w/v] NaCl, 0.5% [w/v] yeast extract; pH 7.4) and on LB agar (LB broth amended with 1.5% [w/v] bacteriological agar). The bacterial cultures were maintained on agar plates at 4°C or at -70°C as glycerol cultures, whilst *R. solanacearum* strains were also stored in sterilized dH_2O at room temperature. When appropriate, *E. coli* growth media were supplemented with ampicillin (50 µg/ml), tetracycline (10 µg/ml) or gentamycin (7 µg/ml), and*R. solanacearum* growth media were supplemented with tetracycline (10 µg/ml) or gentamycin (15 µg/ml). All antibiotics were obtained from Roche Diagnostics (Mannheim, Germany).

3.2.2 DNA isolation and purification

Plasmid DNA was extracted from *E. coli* with a Zyppy Plasmid Miniprep Kit, whilst genomic DNA was isolated from *R. solanacearum* with a ZR Fungal/Bacterial DNA Isolation Kit. Restriction DNA fragments were purified from agarose gels by use of a Zymoclean Gel DNA Recovery Kit. These kits were all obtained from Zymo Research Corporation (Orange, CA, USA) and the procedures were performed in accordance with the manufacturer's instructions. PCR amplicons were purified with the QIAquick® PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

3.2.3 DNA amplification and analysis

3.2.3.1 Primers

Oligonucleotide primers used in PCR assays to amplify selected genomic regions from *R. solanacearum* NB336 were designed using DNAMAN v.4.12 (Lynnon Biosoft, Quebec Canada) on the basis of the published complete genome sequence of *R. solanacearum* strain GMI1000 (Salanoubat *et al*., 2002). To facilitate subsequent cloning procedures involving the PCR-amplified products, unique restriction endonuclease recognition sites were included at the 5' terminus of the different oligonucleotides. The primers, indicated in Table 3.2, were synthesized by Integrated DNA Technologies (Coralville, IA, USA).

3.2.3.2 Polymerase chain reaction (PCR)

Each of the PCR reaction mixtures (25 µl) contained 100 ng of template DNA, 10 pmol of each of the sense and antisense primers, 5% (v/v) DMSO, 1 × PCR buffer (75 mM Tris-HCl [pH 8.8], 16 mM $(NH_4)_2SO_2$, 0.1% [v/v] Tween-20), $MgCl_2$ at 1.5 mM, each deoxynucleotide triphosphate (dNTP) at a concentration of 250 µM and 0.5 U of Biotaq™ DNA polymerase (Bioline Inc., Randolph, MA, USA). The tubes were placed in an Eppendorf MasterCycler® (Eppendorf, Hamburg, Germany) or GeneAmp® 2700 thermal cycler (Applied Biosystems, Foster City, CA). The thermocycling profile consisted of an initial denaturation step at 95°C for 10 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at the appropriate temperature for 30 s and elongation at 72°C for 1 min. After the last cycle, the reactions were kept at 72°C for 7 min to complete synthesis of all DNA strands. For control purposes, reaction mixtures identical to those above were prepared, except that template DNA was omitted. Aliquots of the PCR reaction mixtures were subsequently analyzed by agarose gel electrophoresis.

Table 3.1 Bacterial strains and plasmids used in this study

Strain/plasmid	Relevant characteristics*	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, potato isolate; Swm$^+$, Twt$^+$, HR$^+$, EPS$^+$	CBS, Pretoria
NB336Δ*cpaF2*	NB336::Gm^r; Swm$^+$, Twt$^+$, HR$^+$, Gm^r EPS$^+$	This study
NB336Δ*cpaF2*::*cpaF2*	NB336Δ*cpaF2* with complementation plasmid; Gm^r, Tc^r	This study
Plasmids		
pUC19	Cloning vector; ColE1, Amp^r, LacZα peptide	Stratagene
pUC19-TadABZ	2.3-kb amplicon, comprising full-length *cpaF2* gene (RSP1085) 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-TadABZ-Gent	pUC19-TadABZ with a Gm^r cassette inserted at the *Not*I and *Pst*I sites of the *cpaF2* ORF	This study
pBluescript SKII (+)	Cloning vector; ColE1, Amp^r, LacZα peptide	Stratagene
pLAFR6	Inc P, RK2-derived plasmid vector; Tc^r	Huynh *et al.* (1989)
pLAFR6-LacP	*lac* promoter (235 bp) from pBluescript SKII (+) cloned into the *Hind*III and *Xba*I sites of pLAFR6	This study
pLAFR6-LacP-CpaF2	pLAFR6-LacP with the 1.35-kb *cpaF2* (RSP1085) gene cloned into the *Xba*I and *Eco*RI sites downstream of the *lac* promoter	This study

* Swm, swimming motility; Twt, twitching motility; HR, hypersensitive response on tobacco; EPS, exopolysaccharide slime; Amp^r, Gm^r, Tc^r, resistant to ampicillin, gentamycin and tetracycline, respectively

Table 3.2 Primers used in this study

Primer	Nucleotide sequence*	Restriction site	Tm of primer
TadAZ-F	5' - CGTCGA**gaattc**CGGGCAGTTGC - 3'	*Eco*RI	58°C
TadAB-R	5' - CAACCGCCTGCG**aagctt**CTTCG - 3'	*Hind*III	58°C
Gm-R	5' - GCGCTCGAGTTAGGTGGCGGTACTTGGGTCG - 3'		60°C
CpaF2-F	5' - CGACCAGAGCGC**gaattc**AGACATGGAATC - 3'	*Eco*RI	62°C
CpaF2-R	5' - GGAACGGAAC**tctaga**TGACACAACCCATCG - 3'	*Xba*I	62°C
TadB2-F	5' - GATGGTCGACCAGAGCGTGCCG - 3'		60°C
LacP-F	5' - CGGTA**tctaga**TTTTGTTCCCTTTAGTGAG - 3'	*Xba*I	62°C
LacP-R	5' - TAATGCAGCTGGCACGA**aagctt**TCCC - 3'	*Hind*III	62°C
M13-F	5' - GTAAAACGACGGCCAGT - 3'		55°C
M13-R	5' - GTTTCCCAGTCACGAC - 3'		55°C
CpaF1-F	5' - ATCGCACGGAGCGCATCATGTCACTGC - 3'		62°C
CpaF1-R	5' - ATCCATGATGCCTCTCCCTAGACTTCGTACTG - 3'		62°C

* In primer sequences, the restriction endonuclease sites are indicated in bold lower case letters, while the annealing positions of the respective primers on the *R. solanacearum* genome are shown in Fig. 3.5a.

3.2.3.3 Agarose gel electrophoresis

DNA was analyzed by agarose gel electrophoresis (Sambrook and Russell, 2001). For this purpose, horizontal 1% (w/v) agarose gels were cast and electrophoresed in 1 × TAE buffer (40 mM Tris-HCl, 20 mM NaOAc, 1 mM EDTA; pH 8.5). The agarose gels were supplemented with 0.5 µg/ml ethidium bromide to allow visualization of the DNA on a UVP Model M-15 UV transilluminator (Vilber Lourmat, Paris, France). Where appropriate, DNA fragments were sized according to their migration in the agarose gel as compared to that of standard DNA molecular weight markers, namely Hyperladder™ I (Bioline), and O'RangeRuler™ or FirstRuler™ DNA markers (Fermentas, St. Leon-Rot, Germany).

3.2.4 Nucleotide sequencing

The nucleotide sequence of PCR amplicons and of cloned insert DNA was determined with the ABI-PRISM® BigDye™ Terminator v.3.1 Cycle Sequencing Ready Reaction kit (Applied Biosystems) according to the instructions of the manufacturer. The oligonucleotides used in the sequencing reactions were the same as those used in the PCR reactions (Table 3.2). The sequencing reactions contained 100 ng of purified template DNA, 0.5 µl of BigDye™ Termination Mix, 1 × sequencing buffer, 3.2 pmol of sequencing primer and Sabax™ water to a final volume of 10 µl. Cycle sequencing reactions were performed in a GeneAmp® 2700 thermal cycler (Applied Biosystems) with 25 of the following cycles: denaturation at 96°C for 10 s, annealing at 50°C for 5 s and extension at 60°C for 4 min. The extension products were subsequently precipitated by addition of 20 µl of Sabax™ water, 2 µl of 3 M NaOAc (pH 4.6) and 48 µl of absolute ethanol to each of the sequencing reactions. The tubes were incubated for 15 min at -20°C, centrifuged at 13 000 × *g* for 10 min and the supernatant carefully aspirated. The DNA pellets were rinsed with 70% ethanol, air-dried and then submitted to the University of Pretoria's DNA Sequencing Core Facility for electrophoresis on an ABI PRISM™ 3100 Genetic Analyzer (Applied Biosystems). Nucleotide sequences were analyzed with DNAMAN v.4.12 (Lynnon BioSoft) and BioEdit Sequence Alignment Editor v.7.0.4.1 (Hall, 1999) software programs, and identified by BLAST-N searches against the GenBank database (available at http://www.ncbi.nlm.nih.gov/BLAST).

3.2.5 Cloning of DNA fragments into plasmid vectors

3.2.5.1 Restriction endonuclease digestions

Restriction endonuclease digestions were performed in microcentrifuge tubes in a final volume of 20 µl and contained the appropriate concentration of salt (using the 10× buffer supplied by the manufacturer) for the specific enzyme and 5-10 U of enzyme per µg of DNA. The reaction mixtures were incubated for 2 h at 37°C. All restriction enzymes were supplied by Fermentas. The digestion products were analyzed on a 1% agarose gel in the presence of a DNA molecular weight marker, and the appropriate DNA fragments were purified from the agarose gel and used in ligation reactions.

3.2.5.2 Ligation reactions

Digested PCR amplicons (250 ng) and vector DNA (50 ng) were ligated in a final volume of 20 µl, which contained 1 × DNA ligase buffer (2 mM Tris-HCl, 0.1 mM EDTA, 0.5 mM DTT, 6 mM KCl, 5% [v/v] glycerol; pH 7.5) and 1 U of T4 DNA ligase (1 U/µl; Roche Diagnostics). The ligation reactions were incubated overnight at 16°C.

3.2.5.3 Preparation of competent *E. coli* DH5α cells

Competent *E. coli* DH5α cells were prepared and transformed according to the methods described by Sambrook and Russell (2001). A single colony of a freshly streaked culture of *E. coli* DH5α was inoculated into 100 ml of LB broth and cultured overnight at 37°C with shaking. An aliquot (1 ml) of the overnight culture was then used to inoculate 100 ml of LB broth, pre-warmed to 37°C. The culture was incubated at 37°C with shaking and the optical density (OD) was measured at 600 nm every 30 min until an OD_{600} of 0.5 was reached. The flask was then incubated on ice for 15 min to inhibit further bacterial growth. The cells from 30 ml of the culture were collected by centrifugation in an Eppendorf 5804R centrifuge at 4000 × *g* for 10 min at 4°C. The cell pellet was gently suspended in 10 ml of ice-cold 0.1 M $CaCl_2$ and incubated on ice for 1 h. The cells were harvested, as described above, and suspended in 1 ml of the 0.1 M $CaCl_2$ solution. The competent cells were incubated on ice for 1 h prior to transformation or, alternatively, were stored at -70°C until further use.

3.2.5.4 Transformation of competent cells

To transform the prepared competent *E. coli* DH5α cells, 100 µl of the competent cells was mixed in a pre-chilled microcentrifuge tube with 10 µl of the ligation reaction mixture. After incubation on ice for 1 h, the tubes were subjected to a heat shock by incubating the tubes at 37°C for 10 min. Subsequently, 500 µl of pre-warmed (37°C) LB broth was added to the tubes followed by incubation for 2 h at 37°C. As controls, competent cells were transformed with DNA of a known concentration to determine the transformation efficiency, and untransformed cells were used to test for contamination. Aliquots of the transformation mixtures were subsequently plated onto LB agar supplemented with the appropriate antibiotic. For plasmids containing a *lacZ'* marker gene (pUC19), the cells were plated together with 50 µl of 2% (w/v) X-Gal and 10 µl of 100 mM IPTG. The agar plates were incubated overnight at 37°C.

3.2.6 Plasmid constructions

All molecular cloning techniques employed in the construction of recombinant vectors were performed according to the procedures described in the preceding sections. All plasmid constructions were verified by PCR assays, restriction endonuclease digestion and nucleotide sequencing.

- **Allelic exchange vector pUC19-TadABZ-Gent**

Primers TadAZ-F and TadAB-R were used with genomic DNA of *R. solanacearum* NB336 as template DNA to PCR amplify a 2.303-kb DNA fragment containing 531 bp of the upstream *cpaE2* (RSP1086) gene, the full-length *cpaF2* (RSP1085) gene (1.350 kb) and 422 bp of the downstream *tadB2* (RSP1084) gene. The amplicon was digested with both *Eco*RI and *Hind*III, and then cloned into pUC19 that had been prepared in an identical manner. The recombinant plasmid, designated pUC19-TadABZ, was digested with both *Not*I and *Pst*I, which cuts in the *cpaF2* ORF, and a 1.34-kb gentamycin resistance cassette, recovered from pGEM-Gent (Smith, 2003) by digestion with *Not*I and *Pst*I, was ligated into the deletion site to construct the allelic exchange vector pUC19-TadABZ-Gent. The cloning strategy is indicated diagrammatically in Fig. 3.1.

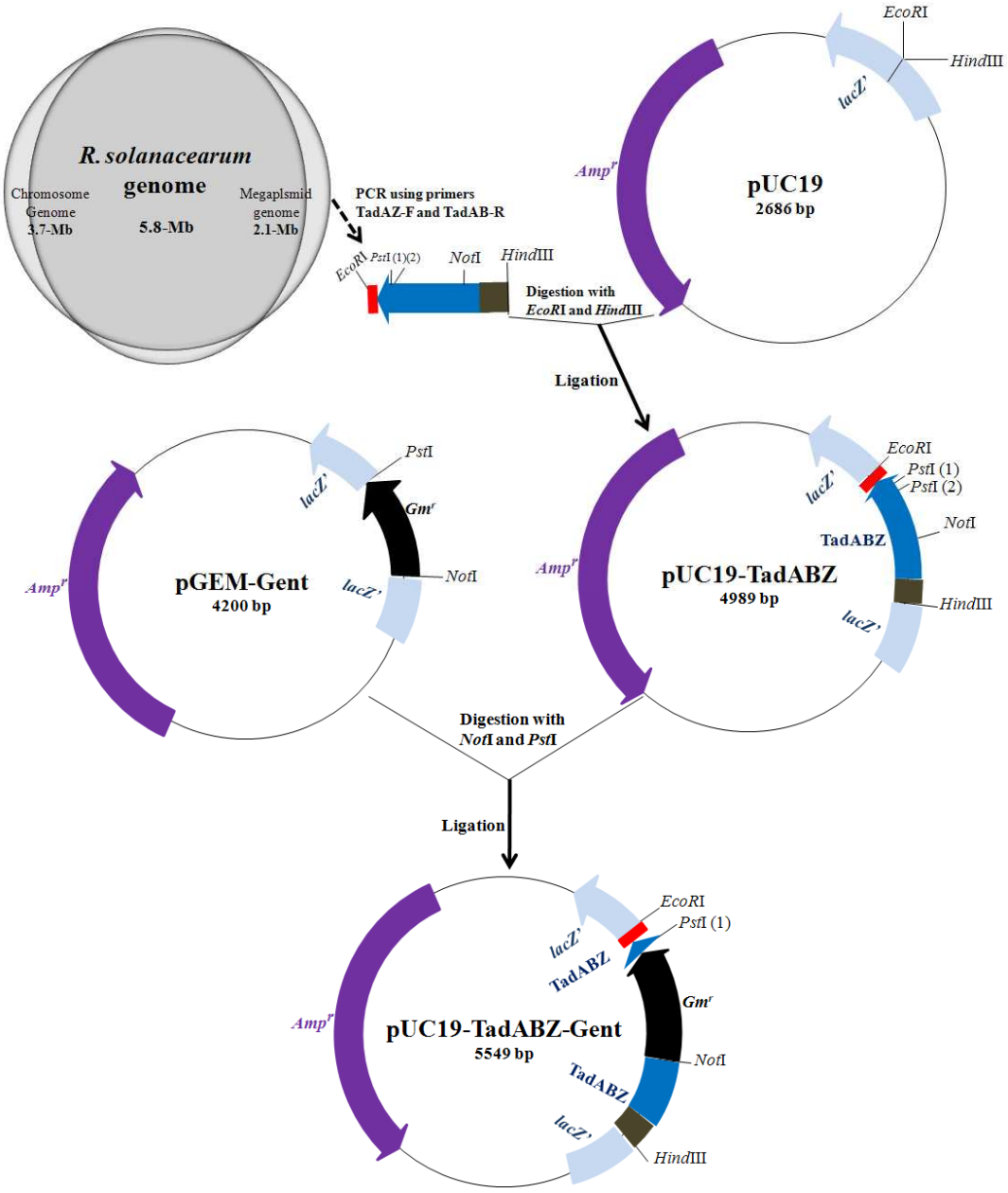


Fig. 3.1 Construction of allelic exchange vector pUC19-TadABZ-Gent, in which the mutated *cpaF2* (RSP1085) gene (blue) is flanked by sequences of the upstream *cpaE2* (RSP1086) gene (red) and downstream *tadB2* (RSP1084) gene (brown).

- **Complementation plasmid pLAFR6-LacP-CpaF2**

Towards construction of the complementation plasmid, the 235-bp *lac* promoter of the plasmid pBluescript SKII (+) was PCR amplified with primers LacP-F and LacP-R, digested with both *Hind*III and *Xba*I and then cloned into identically digested pLAFR6 (Huynh *et al.*, 1989). The recombinant plasmid was designated pLAFR6-LacP. Subsequently, the 1.35-kb *cpaF2* gene (RSP1085) was PCR amplified by making use of primers CpaF2-F and CpaF2-R together with genomic DNA from *R. solanacearum* NB336 as template DNA. The amplicon was digested with both *Eco*RI and *Xba*I and cloned into identically digested pLAFR6-LacP plasmid DNA to generate the complementation plasmid pLAFR6-LacP-CpaF2. The cloning strategy is indicated diagrammatically in Fig. 3.2.

3.2.7 Construction of a *cpaF2* mutant *R. solanacearum* strain

Competent *R. solanacearum* NB336 cells were prepared and transformed with the allelic exchange vector pUC19-TadABZ-Gent according to the procedures described previously (Section 3.2.5.3), except that the transformation mixture was incubated overnight at 30°C prior to plating onto TZC agar supplemented with 15 µg/ml of gentamycin. Following incubation for 48-72 h at 30°C, single colonies were selected from the agar plates and maintained on TZC agar containing gentamycin. One of these, designated *R. solanacearum* NB336Δ*cpaF2*, was selected and used in subsequent investigations.

3.2.8 Characterization of the *R. solanacearum* NB336Δ*cpaF2* strain

The replacement of the wild-type *cpaF2* gene with the mutant allele in the selected *R. solanacearum* strain was verified by Southern blot analysis (Southern, 1975) and PCR analyses.

3.2.8.1 Southern blot analysis

- **Preparation of the labelled probe**

For Southern blot analysis, the gentamycin resistance cassette was used as probe and labelled with digoxigenin-dUTP (DIG-dUTP) by making use of the DIG-High Prime DNA Labelling and Detection Starter kit (Roche Diagnostics) according to the manufacturer's instructions. The gentamycin resistance cassette was PCR amplified from plasmid pGEM-Gent with primers M13-F and M13-R, as described previously (Section 3.2.3.2).

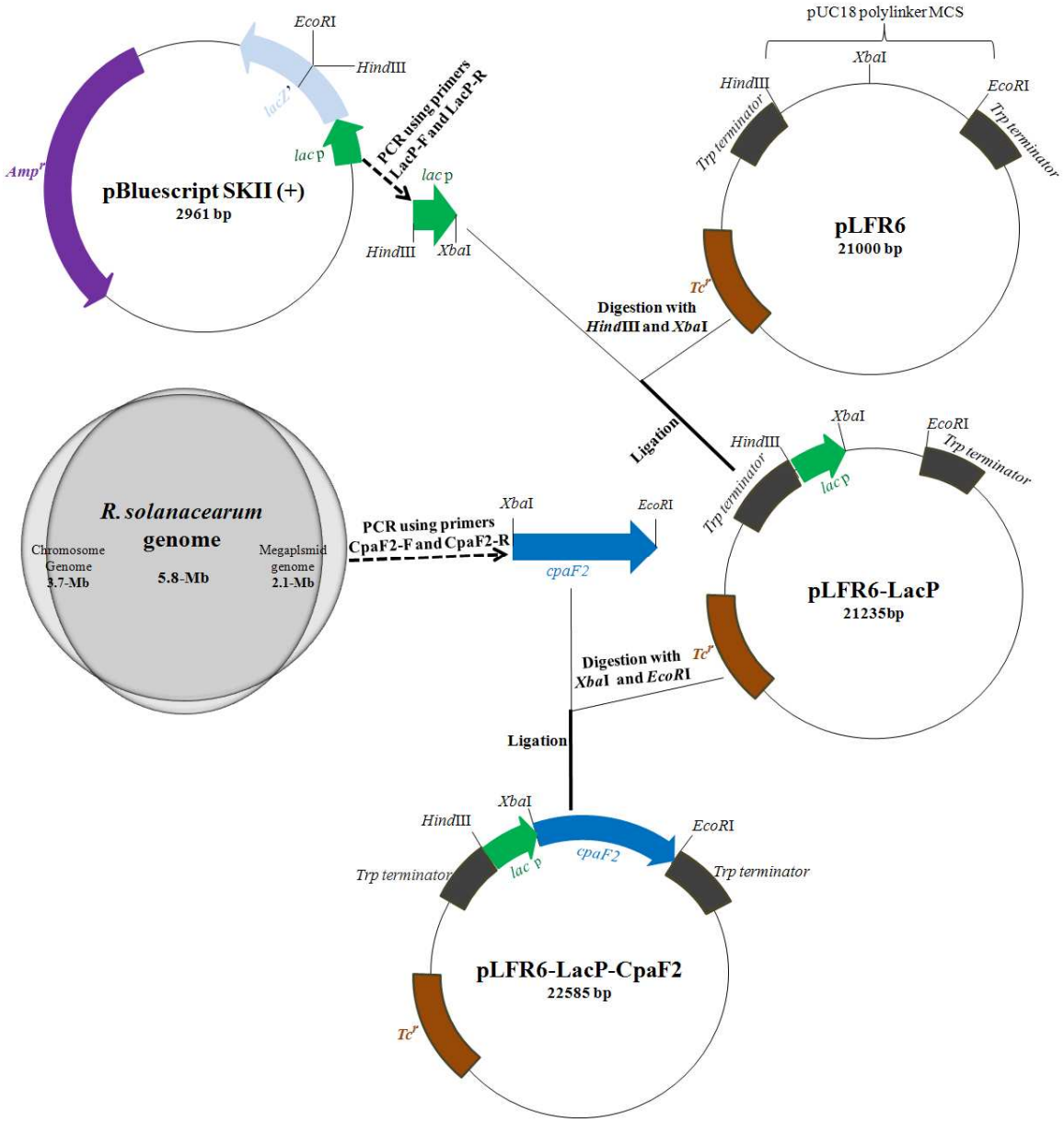


Fig. 3.2 Construction of complementation plasmid pLFR6-LacP-CpaF2.

To prepare the DIG-dUTP labelled probe, 500 ng of the purified amplicon was diluted with Sabax™ water to a final volume of 16 µl. The DNA was denatured by boiling in a water bath for 10 min and then chilled in an ice water bath. The labelling reaction was initiated by addition of 4 µl of DIG-High Prime labelling mix (containing random primers, nucleotides, DIG-dUTP, Klenow polymerase and buffer components) and then incubated for 20 h at 37ºC. Following incubation the reaction was terminated by addition of 2 µl of 0.2 M EDTA (pH 8) and heating to 65ºC for 10 min. The DNA probe concentration was quantified using the labelled control DNA provided with the kit according to the manufacturer's instructions.

- **Preparation of the membrane**

Genomic DNA of the *R. solanacearum* wild-type NB336 and mutant NB336Δ*cpaF2* strains was digested with *Hind*III before being resolved by electrophoresis on a 1% (w/v) agarose gel. The PCR amplified gentamycin resistance cassette was included in the analysis as a control to confirm probe specificity. The DNA fragments were transferred from the agarose gel to a Hybond™-N nylon membrane (GE Healthcare Bio-Sciences, Uppsala, Sweden) by capillary blotting. For this purpose, the DNA was first denatured by soaking the gel for 30 min with constant agitation in denaturing solution (1.5 M NaCl, 0.5 M NaOH), rinsed in dH_2O and then neutralized, as above, in neutralization solution (1 M Tris-HCl [pH 7.2], 1.5 M NaCl). Six pieces of filter paper, soaked in 20 × SSC (3 M NaCl, 0.3 M sodium citrate; pH 7.0), were stacked in a shallow container. The inverted gel was then placed onto the filter paper, followed by the nylon membrane and then four pieces of filter paper, all of which were pre-soaked in 2 × SSC. Two additional dry filter papers and several paper towels were stacked on top of the wet filter papers and weighed down by a light weight. Transfer was allowed to proceed at room temperature for at least 18 h, after which the DNA fragments were cross-linked to the membrane by UV irradiation for 5 min on each side on a transilluminator.

- **Hybridization**

The hybridization temperature was calculated using the following formula (Roche Diagnostics): $T_m = 49.82 + 0.41(\%G+C) - (600/l)$, where l is the length of the hybrid in bp. The optimum hybridization temperature was then calculated using $T_{opt} = T_m - 25°C$. The optimal hybridization temperature for the gentamycin probe was calculated to be 42°C. The membrane was incubated with gentle agitation for 30 min in 10 ml of DIG-Easy Hyb buffer, pre-heated to 37°C. The pre-hybridization buffer was decanted and replaced with 10 ml of

DIG-Easy Hyb buffer containing the labelled DNA probe at a concentration of 25 ng/ml. The DNA probe was denatured, prior to its addition to the hybridization buffer, by boiling in a water bath for 5 min and rapidly cooling in an ice water bath. Hybridization was performed at 42°C for 4 h, after which the membrane was washed twice for 5 min each in 2 × SSC, 0.1% SDS at room temperature, followed by two washes of 15 min each in 0.5 × SSC, 0.1% SDS at 60°C.

- **Detection of the hybridized DNA probe**

To detect the hybridized DNA probe the membrane was rinsed in washing buffer (0.1 M maleic acid, 0.15 M NaCl, 0.3% [v/v] Tween-20; pH 7.5), followed by a 30-min incubation in blocking solution (supplied in the kit and prepared according to the manufacturer's instructions). The membrane was then incubated for 30 min at room temperature in 20 ml of an antibody solution, comprising a 1:5 000 dilution of the alkaline phosphatase-conjugated anti-digoxygenin antibody in fresh blocking solution. The membrane was washed twice for 15 min each in washing buffer, followed by equilibration for 5 min in detection buffer (0.1 M Tris-HCl, 0.1 M NaCl; pH 9.5). The detection buffer was discarded and the membrane was then immersed in 10 ml of freshly prepared alkaline phosphatase enzyme substrate solution (NBT/BCIP stock diluted 1:50 in detection buffer). Once the bands became visible, the color reaction was stopped by rinsing the membrane in dH_2O.

3.2.8.2 PCR analyses

The *R. solanacearum* NB336Δ*cpaF2* strain was analyzed for the presence of the gentamycin resistance cassette within the *cpaF2* gene by PCR analyses. For this purpose, primers CpaF2-R and Gm-R were used to amplify a hybrid amplicon consisting of the 3'-end of the gentamycin resistance cassette and the 5'-end of the interrupted *cpaF2* gene, whereas primer pairs CpaF2-F and CpaF2-R, as well as CpaF2-R and TadB2-F (Table 3.2) were used to amplify the *cpaF2* gene interrupted by the gentamycin resistance cassette in mutant genomic DNA. The PCR reactions were performed, as described previously (Section 3.2.3.2), except that primer annealing was performed at 60°C for 30 s for all primer pairs. For all of the analyses, Sabax™ water served as a negative control, while chromosomal DNA extracted from the parental and mutant strains provided sample template DNA. Following PCR amplification, aliquots of the respective reaction mixtures were analyzed by agarose gel electrophoresis and their identity confirmed by nucleotide sequencing.

3.2.9 Complementation of the *R. solanacearum* NB336Δ*cpaF2* mutant strain

The complementation plasmid pLAFR6-LacP-CpaF2 was introduced into the *R. solanacearum* NB336Δ*cpaF2* mutant strain by electroporation. Electrocompetent *R. solanacearum* NB336Δ*cpaF2* cells were prepared, as described previously (Allen *et al.*, 1991). Briefly, an overnight culture was diluted 1:50 into 100 ml of fresh CPG broth and incubated at 30°C until an OD_{600} of 0.6 was reached. The flask was incubated on ice for 20 min to inhibit further bacterial growth. The bacterial cells were harvested by centrifugation at 5000 × *g* for 5 min and the cell pellet was suspended in 50 ml of ice-cold 10% (v/v) glycerol. This step was repeated three times, except that the cell pellet was suspended in 25 ml, 5 ml and 500 µl of the ice-cold 10% (v/v) glycerol solution, respectively. For electroporation, the cells (100 µl) were mixed with 5 µg of purified pLAFR6-LacP-CpaF2 plasmid DNA. The cells were transferred into a 0.1-cm inter-electrode gap electroporation cuvette (Eppendorf) and exposed to a single electrical pulse using an Eppendorf Multiporator® set at 2000 V. Immediately following the electrical discharge, 100 µl of CPG broth was added to the electroporation cuvette and incubated on the bench for 20 min. The contents of the cuvette was subsequently transferred to a sterile test tube containing 800 µl of CPG broth and incubated for 16 h at 30°C with gentle agitation. Aliquots of the electroporated cells were plated onto TZC agar containing both gentamycin and tetracycline, and then incubated for 48 h at 30°C.

3.2.10 Phenotypic characterization of *R. solanacearum* NB336Δ*cpaF2*

3.2.10.1 Growth curves

The *R. solanacearum* wild-type NB336, mutant NB336Δ*cpaF2* and complemented mutant NB336Δ*cpaF2*::*cpaF2* strains were cultured overnight at 30°C with shaking in 10 ml of CPG broth supplemented with the appropriate antibiotics. The overnight cultures were diluted 1:50 in fresh broth and incubated until mid-exponential phase was reached (OD_{600} = 0.5). The cultures were subsequently used to inoculate 200 ml of CPG broth to an OD_{600} of 0.03 and the flasks were incubated at 30°C with shaking. The OD_{600} reading of each culture was determined every 2 h for the first 10 h and again between 20 h and 30 h with a Spectronic® 20 Genesys™ spectrophotometer (Spectronics Corporation, New York, NY, USA). Three independent assays were performed for each bacterial strain.

3.2.10.2 Scanning electron microscopy (SEM)

The cells of stationary phase-cultures (24 h) of the *R. solanacearum* wild-type, mutant and complemented mutant strains were collected by centrifugation at 4000 × *g* for 5 min and then fixed for 1 h at room temperature in fixing solution (2.5% gluteraldehyde in 0.075 M phosphate buffer). The fixed cells were subsequently filtered through a 0.2-µm filter (Millipore Corporation, Billerica, MA, USA) and washed three times for 10 min each with 0.075 M phosphate buffer (pH 7.4), before being dehydrated by sequential treatment for 10 min each in 30%, 50%, 70%, 90% and 100% ethanol. The treatment with 100% ethanol was repeated twice more to ensure complete dehydration of the samples. The filters were then placed onto grids, scatter-coated with gold and observed on a JSM 5800 scanning electron microscope (JEOL, Tokyo, Japan) at 10 kV.

3.2.10.3 Quantification of extracellular polysaccharides (EPS)

The method described by Jang *et al.* (2005) was used to quantify EPS. The *R. solanacearum* wild-type, mutant and complemented mutant strains were cultured in 50 ml of LB broth at 30°C for 96 h. The cultures were subsequently standardized to an OD_{600} of 0.5, centrifuged at 5000 × *g* for 20 min at 4°C and the supernatants were carefully removed and stored at 4°C. The cell pellets were washed twice with 0.1 M NaCl, suspended in 500 µl of dH_2O and stored at -20°C overnight. To recover EPS, culture supernantants were adjusted to 0.1 M NaCl and four volumes of absolute ethanol were added followed by incubation overnight at 4°C. The precipitated material was recovered by centrifugation (5000 × *g*, 10 min, 4°C), dissolved in 1 ml of dH_2O, heated at 65°C for 15 min and then centrifuged for 20 min to remove insoluble material. The concentration of hexosamines, which is a reliable indication of EPS content (Brumbley and Denny, 1990), was estimated using a modified Elson-Morgan reaction (Jang *et al.*, 2005). EPS samples (500 µl) were placed in screw-capped tubes and 200 µl of 6 M HCl was added slowly to dissolve the EPS polymer. The tubes were purged with liquid nitrogen before being closed tightly. Standard solutions of galactosamine hydrochloride (10-100 µg/ml) were heat-treated simultaneously. The hydrolysis was carried out in an autoclave at 115°C for 1 h. The hydrolysis reaction was terminated by cooling the samples in an ice water bath and neutralized with an equal volume (200 µl) of 6 M NaOH. The neutralized samples and standard solutions were mixed with 500 µl of freshly prepared acetylacetone reagent (4.8% [v/v] acetylacetone in equal volumes of 1 M $NaHCO_3$ and 1 M Na_2CO_3; pH 9.6). The solutions were autoclaved at 105°C for 15 min, after which acetylation was terminated by

cooling the samples in an ice water bath. Subsequently, 2.5 ml of absolute ethanol, followed by 0.5 ml of Ehrlich reagent (0.8 g *p*-dimethylaminobenzaldehyde in 30 ml each of absolute ethanol and concentrated [35%] HCl) was added. The tubes were placed at 65°C for 10 min, cooled to room temperature and the absorbance was measured at 530 nm. The amounts of total hexosamines liberated during hydrolysis were calculated from the standard plot between A_{530} and galactosamine concentrations (10-100 µg/ml).

3.2.10.4 Motility assays

Flagella-dependent swimming motility was assayed as described by Liu *et al.* (2001). Standardized cultures (OD_{600} = 0.1) of the *R. solanacearum* wild-type, mutant and complemented mutant strains were stab inoculated into 3-mm-thick motility agar (1% [w/v] tryptone, 0.3% [w/v] agar). The agar plates were incubated for 48 h at 30ºC and the zone of colonization was subsequently compared. T4ap-mediated twitching motility was assayed as described by Liu *et al*. (2001). For this purpose, standardized cultures (OD_{600} = 0.1) of the respective bacterial strains were inoculated onto fresh CPG agar and incubated 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.

3.2.10.5 Biofilm assay

Biofilm formation on an abiotic surface was determined using a quantitative plate assay, as described by Yao and Allen (2007). A 96-well polyvinyl chloride (PVC) plate (Nunc, Roskilda, Denmark), containing 50 µl of CPG broth, was inoculated with 150 µl of standardized cultures (OD_{600} = 0.1) of the *R. solanacearum* wild-type, mutant and complemented mutant strains. The plates were sealed with plastic wrap and incubated at 30ºC for 48 h under static conditions. Following incubation, the cultures were aspirated and the wells rinsed three times with sterile dH_2O to remove unattached cells. The plates were dried at 65ºC for 20 min, before staining with 300 µl of a 1% (w/v) solution of crystal violet (Sigma-Aldrich, St. Louis, MO, USA). Following incubation at room temperature for 20 min, the wells were rinsed three times with water to remove excess stain and then filled with 300 µl of methanol prior to measuring the optical density at 600 nm. Three independent assays were performed for each bacterial strain.

3.2.11 Plant assays

3.2.11.1 *In planta* growth curves

For determination of *in planta* growth curves, mid-exponential phase cultures (OD_{600} = 0.5) of the *R. solanacearum* wild-type NB336 and mutant NB336Δ*cpaF2* strains were inoculated to an OD_{600} of 0.1-0.2 in sterile dH_2O. An aliquot of the standardized bacterial cultures (10^8 CFU/ml) were then injected into the stems of 30-day old potato plants (*Solanum tuberosum* BP1) and incubated in a greenhouse at 28-30°C. A 3-cm segment of the stem, obtained from 1 cm above the point of inoculation, was excised at each time interval and surface sterilized by rinsing the stems in dH_2O and then in 70% ethanol. The stems were then rinsed with dH_2O and macerated in 5 ml of sterile dH_2O with a mortar and pestle. Serial dilutions of the stem homogenates were prepared in sterile dH_2O and plated in triplicate onto TZC agar containing the appropriate antibiotic. The agar plates were incubated for 48 h at 30°C, after which colonies were counted on plates containing between 30 to 300 colonies and then averaged.

3.2.11.2 Potato root colonization assay

Quantitative root attachment assays were performed according to the method of Anderson and Guerra (1985). Healthy 30-day old potato plants were inoculated by drenching 0.5 kg of soil with 50-ml suspensions of the *R. solanacearum* wild-type, mutant and complemented mutant strains (1×10^8 CFU/ml). After 15 days in a greenhouse at 28-30°C, the potato plants were removed from their pots and the roots were rinsed with sterile dH_2O and blotted lightly on absorbent paper. Then, one set of roots was excised and prepared for scanning electron microscopy (Section 3.2.10.2). A matched set of potato roots was excised, weighed and 2 g of material was ground in 10 ml of sterile dH_2O. A dilution series was prepared, as described above, and plated onto TZC agar to quantify the total bacteria adhering to roots. The cell numbers were normalized to root fresh weight. The assay was repeated three times for each *R. solanacearum* strain, and a negative control (sterile dH_2O) was also included.

3.2.11.3 Survival of *R. solanacearum* strains in soil

Five ml of bacterial suspensions of the *R. solanacearum* wild-type, mutant and complemented mutant strains (1×10^8 CFU/ml) was inoculated into 25 g of potting soil in triplicate glass beakers for sampling on days 0, 5 and 15 after inoculation. The inoculated soil was placed in a greenhouse at 28-30°C. At each sampling date, the soil samples were transferred into sterile

plastic bags and thoroughly mixed before weighing out a 10-g sub-sample. The soil sub-samples were mixed with 10 ml of sterile dH_2O and serial dilutions were prepared. From the dilutions, 0.1-ml volumes were plated onto TZC agar and the agar plates were incubated for 48 h at 30°C. The assay was repeated three times for each *R. solanacearum* strain, and a negative control (sterile dH_2O) was also included.

3.2.11.4 Potato pathogenicity trials

Virulence tests of the *R. solanacearum* wild-type NB336, mutant NB336Δ*cpaF2* and complemented mutant strains were conducted on potato plants, as described by Liu *et al*. (2001). The plants were not watered for 24 h prior to inoculation (Williamson *et al*., 2002). Subsequently, potato plants (30-day old, 15 cm in height) were inoculated by drenching the soil in 20-cm pots with 50 ml of the bacterial suspension (1×10^8 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 as follows: 0 = healthy plant, 1 = one leaf wilted, 2 = 2-50% of leaves wilted, 3 = 75% of leaves wilted, and 4 = 100% of leaves wilted (Fock *et al*., 2000; 2001).

To test for elicitation of the defensive hypersensitive reaction (HR), standardized cultures (1×10^8 CFU/ml) of the respective *R. solanacearum* strains were infiltrated into leafs of tobacco (a non-host plant), as described by Liu *et al*. (2001). Sterile dH_2O and *Xanthomonas campestris* were included in the assay as a negative and positive control, respectively. For infiltration, a hole was made in the leaf by pressing the blunt end of a syringe against the leaf surface while supporting the other side of the leaf with a finger, and gently injecting a small volume of inoculum. The plants were observed daily for 7 days.

3.2.12 Statistical analyses

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

3.3 RESULTS

3.3.1 Construction of allelic exchange vector pUC19-TadABZ-Gent

To obtain the *cpaF2* (RSP1085) gene, together with flanking regions to facilitate homologous recombination, primers TadAZ-F (containing an *Eco*RI site) and TadAB-R (containing a *Hind*III site) were used in a PCR with genomic DNA of *R. solanacearum* NB336. An aliquot of the reaction mixture was analyzed by agarose gel electrophoresis and a single discreet amplicon of *ca.* 2.3 kb was observed (Fig. 3.3b, lane 2). The amplicon corresponded in size to the full-length *cpaF2* gene (1.350 kb), flanked by 531 bp of the 3'-end of the upstream *cpaE2* gene and 422 bp of the 5'-end of the downstream *tadB2* gene. In contrast, no amplification products were observed in the negative control in which template DNA was omitted.

Towards construction of the desired allelic exchange vector the purified amplicon was digested with both *Eco*RI and *Hind*III, and ligated into similarly prepared pUC19 vector DNA. Following transformation of competent *E. coli* DH5α cells, recombinant transformants with a Lac^- phenotype were selected from X-gal - containing indicator plates and cultured in LB broth supplemented with ampicillin. The extracted plasmid DNA was analyzed by agarose gel electrophoresis. Plasmid DNA migrating slower than the parental pUC19 vector DNA were selected and analyzed for the presence of a cloned insert DNA by restriction enzyme digestion. Digestion of recombinant plasmid DNA with both *Eco*RI and *Hind*III yielded DNA fragments of *ca.* 2.7 and 2.3 kb, which is in agreement with the expected size of the pUC19 vector and insert DNA, respectively (Fig. 3.3b, lane 5). A recombinant clone, designated pUC19-TadABZ, was selected and the integrity of the cloned insert DNA was verified by nucleotide sequencing of both terminal ends.

Recombinant plasmids pGEM-Gent (Smith, 2003) and pUC19-TadABZ served as sources for the construction of the allelic exchange vector pUC19-TadABZ-Gent (Fig. 3.3a). Digestion of recombinant plasmid pUC19-TadABZ with *Pst*I and *Not*I, which respectively cuts twice and once in the *cpaF2* gene, yielded three DNA fragments of 4.253, 0.440 and 0.321 kb. The 4.3-kb DNA fragment was excised from the agarose gel and purified. This DNA fragment harbors the *cpaF*2 gene from which the Asp and His boxes, as well as Walker B motif, essential for NTPase functioning (Whitchurch *et al.*, 1991; Possot and Pugsely, 1994), has been deleted. The 1.34-kb gentamycin resistance cassette was subsequently recovered from plasmid pGEM-Gent by digestion with *Pst*I and *Not*I, purified from the agarose gel and ligated into the

deletion site of pUC19-TadABZ. Following transformation of competent *E. coli* DH5α cells, plasmid DNA from gentamycin-resistant transformants were characterized first by agarose gel electrophoresis and then by restriction enzyme digestion. Digestion of the recombinant plasmid DNA with both *Eco*RI and *Hind*III yielded DNA fragments of 2.863 and 2.686 kb, which were difficult to resolve on the agarose gel due to their similar sizes (Fig. 3.3b, lane 7). Therefore, the recombinant plasmid DNA was digested with both *Pst*I and *Not*I and resulted in DNA fragments of 4.3 and 1.4 kb (Fig. 3.3b, lane 8). These results thus confirmed that the gentamycin resistance cassette had been cloned successfully. A recombinant clone, designated pUC19-TadABZ-Gent, was selected for further use.

3.3.2 Engineering of an CpaF2 mutant *R. solanacearum* strain

3.3.2.1 Generation of mutant strains

A mutant of the wild-type *R. solanacearum* NB336 strain was generated by introducing the allelic exchange vector pUC19-TadABZ-Gent into the wild-type NB336 strain, and selecting for subsequent homologous recombination events between the *R. solanacearum* DNA flanking the gentamycin resistance cassette in the vector and the wild-type locus on the megaplasmid genome. Recipient *R. solanacearum* strains harbouring an integrated copy of the *cpaF2*::*Gm*^{r} mutant allele were selected by plating onto selective agar medium, as described under Materials and Methods (Section 3.2.7). A gentamycin-resistant *R. solanacearum* strain was selected and designated *R. solanacearum* NB336Δ*cpaF2*.

3.3.2.2 Southern blot analysis of *R. solanacearum* NB336Δ*cpaF2*

To determine whether the gentamycin resistance cassette was present in the genome of the mutant strain, Southern blot analysis was performed. The genomic DNA of *R. solanacearum* NB336Δ*cpaF2* was isolated, digested with *Hind*III and separated by agarose gel electrophoresis. The DNA fragments were transferred onto a nylon membrane by capillary blotting and the membrane was then hybridized with a DIG-dUTP - labelled DNA probe specific for the gentamycin resistance cassette. In this analysis, the PCR amplified gentamycin resistance cassette from plasmid pGEM-Gent was included as a positive control, while *Hind*III-digested genomic DNA of the wild-type *R. solanacearum* NB336 strain was included as a negative hybridization control. Since *R. solanacearum* possesses two *tad* loci that each contains an NTPase-encoding gene, it was of importance to determine which of these loci harbored the *cpaF2*::*Gm*^{r} mutant allele. Consequently, the NTPase-encoding genes

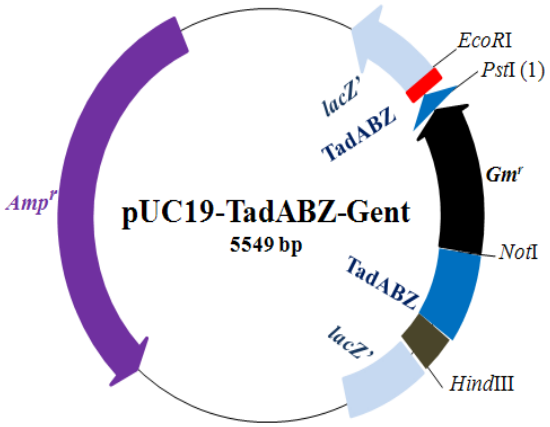


Fig. 3.3a Plasmid map of allelic exchange vector pUC19-TadABZ-Gent.

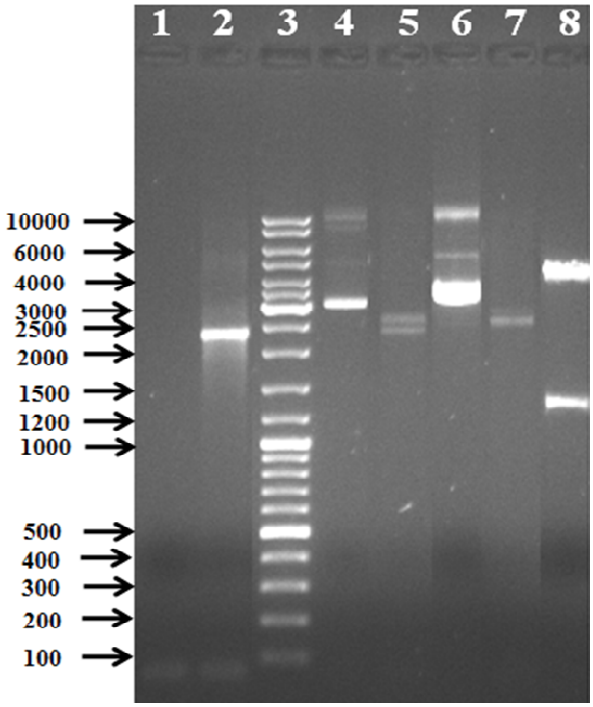


Fig. 3.3b Characterization of allelic exchange vector pUC19-TadABZ-Gent by restriction enzyme digestion. Lane 1, negative control PCR reaction mixture lacking template DNA; lane 2, amplicon obtained by PCR amplification using *R. solanacearum* NB336 genome DNA as template and primers TadAZ-F and TadAB-R; lane 3, DNA molecular weight marker; lane 4, uncut recombinant vector pUC19-TadABZ; lane 5, recombinant vector pUC19-TadABZ digested with both *Eco*RI and *Hind*III; lane 6, uncut vector pUC19-TadABZ-Gent; lane 7, recombinant vector pUC19-TadABZ-Gent digested with *Eco*RI and *Hind*III; lane 8, recombinant vector pUC19-TadABZ-Gent digested with *Not*I and *Pst*I. The sizes of the DNA molecular weight marker in base pairs, O'GeneRuler™ 100-bp DNA Ladder Plus (Fermentas), are indicated to the left of the figure.

cpaF1 and *cpaF2* present in the *tad* loci of the chromosome and megaplasmid genomes, respectively, were PCR amplified and also included in the analysis.

The results (Fig. 3.4) indicated that the probe specific for the gentamycin resistance cassette hybridized to the PCR amplified gentamycin resistance cassette, as well as to a DNA restriction fragment from the *R. solanacearum* NB336Δ*cpaF2* genomic DNA. Importantly, the probe also hybridized to the *cpaF2* (RSP1085) gene PCR amplified from the megaplasmid genome of *R. solanacearum* NB336Δ*cpaF2*, but not with the *cpaF1* (RSC0652) gene amplified from the chromosome genome. The probe neither hybridized to the digested chromosomal DNA of the *R. solanacearum* NB336 strain nor with the PCR amplified *cpaF1* and *cpaF2* genes of this wild-type strain. These results therefore indicated that a single copy of the *cpaF2*::*Gm*^{r} mutant allele was integrated into the megaplasmid genome of *R. solanacearum* NB336Δ*cpaF2*.

3.3.2.3 PCR analyses of *R. solanacearum* NB336Δ*cpaF2*

To confirm the location of the integrated mutant allele in the *R. solanacearum* NB336Δ*cpaF2* strain and to determine whether integration occurred by means of a single or double crossover event, PCR analyses were performed using different pairs of oligonucleotide primers (Fig. 3.5a). These amplified hybrid products only if the gentamycin resistance cassette was located within the mutated *cpaF2* gene of the megaplasmid genome. Moreover, oligonucleotides were also used that annealed to genomic sequences flanking the region in which the mutant allele was integrated.

Primers CpaF2-F and CpaF2-R, which anneal to the 3'- and 5'-end of the *cpaF2* gene, respectively, were used to amplify either a 1.35-kb product in the absence of the gentamycin resistance cassette, or a 1.94-kb product in the presence of the 1.34-kb cassette. A 1.94-kb product was produced when *R. solanacearum* NB336Δ*cpaF2* genomic DNA was used as template, but template DNA of wild-type *R. solanacearum* NB336 generated the 1.35-kb product (Fig. 3.5b). Moreover, primers CpaF2-R and Gm-R were used to amplify a 1.674-kb hybrid product if the gentamycin resistance cassette was located within the mutated *cpaF2* (RSP1085) gene. The product was produced when *R. solanacearum* NB336Δ*cpaF2* genomic DNA was used as template. As expected, when wild-type *R. solanacearum* NB336 genomic DNA was used as template in the PCR reactions no products were amplified with this primer

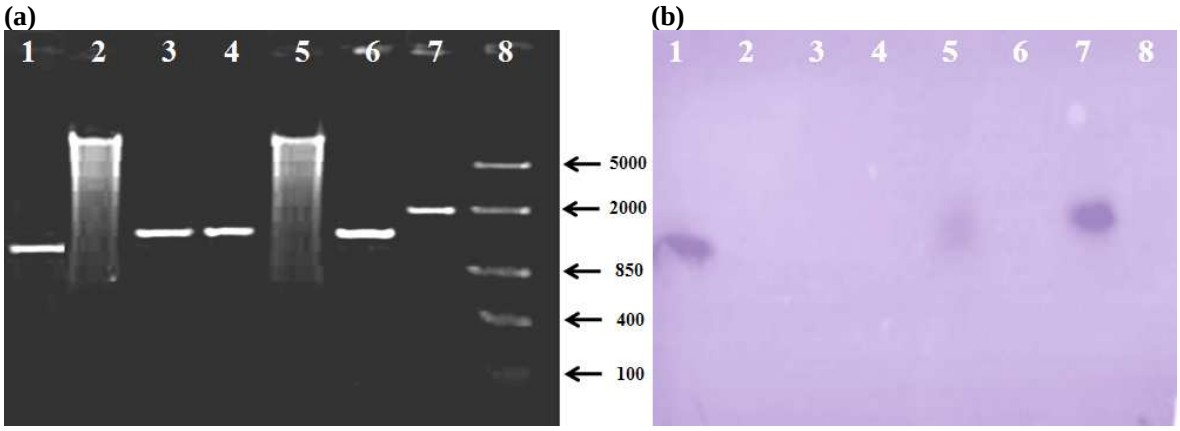


Fig. 3.4 Southern blot analysis of genomic DNA extracted from the *R. solanacearum* mutant strain NB336Δ*cpaF2*, containing a mutated *cpaF2* gene. Genomic DNA extracted from the wild-type NB336 (lanes 2) and mutant NB336Δ*cpaF2* (lanes 5) strains were digested with *Hind*III, resolved by agarose electrophoresis (a) and transferred to a nylon membrane for Southern blotting (b). The membrane was probed with a DIG-labelled gentamicin resistance cassette. An amplicon of the gentamycin resistance cassette (lanes 1) was included as a positive hybridization control. Amplicons of the chromosomal *cpaF1* (RSC0652) and megaplasmid *cpaF2* (RSC1085) genes of the wild-type NB336 strain (lanes 3 and 4, respectively), as well as those of the mutant NB336Δ*cpaF2* strain (lanes 6 and 7, respectively) were also included in the analysis. The sizes of the DNA molecular weight marker in base pairs (lanes 8), FirstRuler™ Middle Range DNA Ladder (Fermentas), are indicated to the right of the Fig. 3.4a.

pair (Fig. 3.5c). In the final analysis, primers were used that annealed to genomic sequences downstream of the *cpaF2* gene in which the mutant allele was integrated. Thus, primers CpaF2-R and TadB2-F were used to amplify either a 2.274-kb or a 2.863-kb product in the absence or presence of the 1.34-kb gentamycin resistance cassette, respectively. As expected, a 2.863-kb product was produced when *R. solanacearum* NB336Δ*cpaF2* genomic DNA was used as template, whilst template DNA from wild-type *R. solanacearum* NB336 generated the 2.274-kb product (Fig. 3.5d). Cumulatively, these results therefore confirmed that a single copy of the *cpaF2*::*Gm*^{r} mutant allele was integrated into the megaplasmid genome of *R. solanacearum* NB336Δ*cpaF2*, and that it occurred by means of a double crossover event.

3.3.3 Construction of complementation plasmid pLAFR6-LacP-CpaF2

To determine whether any altered phenotypes that may be displayed by the *R. solanacearum* NB336Δ*cpaF2* mutant strain was due to mutagenesis of the wild-type *cpaF2* gene, a complementation plasmid, containing a wild-type copy of the *cpaF2* gene under transcriptional control of a *lac* promoter, was constructed whereby the mutant strain could be complemented. Since complementation studies would require that the plasmid DNA is capable of replicating in *R. solanacearum*, the broad-host-range plasmid pLAFR6 was selected for construction of the complementation plasmid. This plasmid vector contains a RK2 replicon that permits replication in a wide variety of Gram-negative bacteria, including *E. coli* and *R. solanacearum*. In addition, the plasmid vector also harbors a tetracycline resistance marker to allow for plasmid selection and maintenance in the respective bacterial hosts (Huynh *et al.*, 1989).

The *lac* promoter was obtained by PCR amplification using primers LacP-F (containing a *Xba*I site) and LacP-R (containing a *Hind*III site) and pBluescript SKII (+) plasmid DNA as template. Following PCR, the 235-bp amplicon was digested with both *Xba*I and *Hind*III, and cloned into the pLAFR6 plasmid vector to generate pLAFR6-LacP. To complete construction of the complementation plasmid, the 1.35-kb *cpaF2* gene, obtained by PCR amplification using primers CpaF2-F (containing an *Eco*RI site) and CpaF2-R (containing a *Xba*I site) and genomic DNA of *R. solanacearum* NB336 as template, was digested with both *Eco*RI and *Xba*I and cloned into pLAFR6-LacP that had been prepared identically. The complementation plasmid, designated pLAFR6-LacP-CpaF2 (Fig. 3.6a), thus contained the *lac* promoter (*lacP*) in the correct transcriptional orientation relative to the *cpaF2* gene.

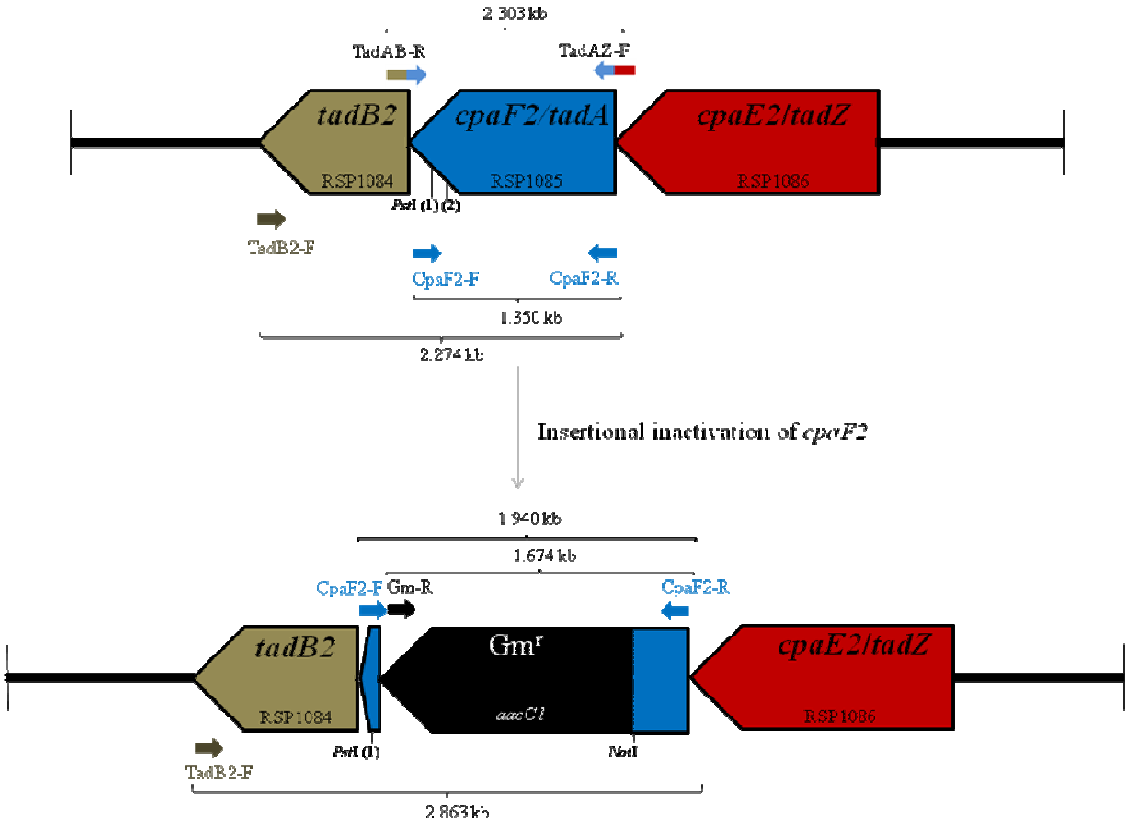
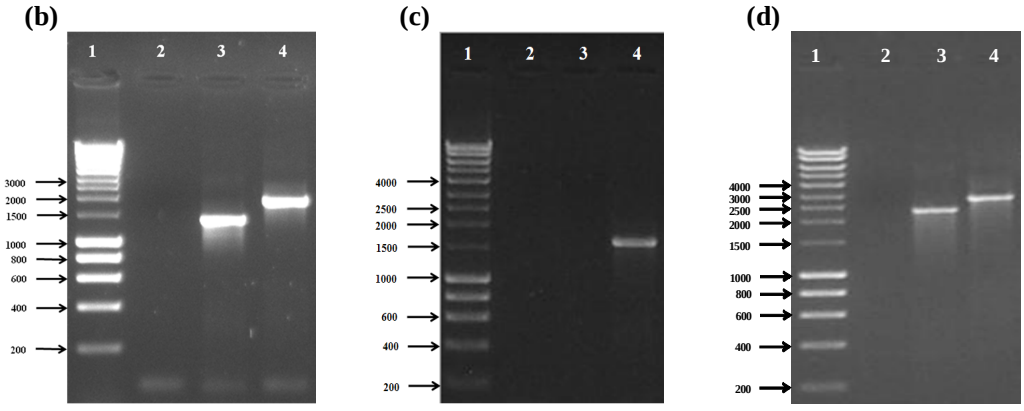


Fig. 3.5a Schematic representation of a portion of the *tad* locus on the megaplasmid genome of *R. solanacearum*, indicating the primer annealing positions and direction of amplification in the wild-type (top panel) and mutant (lower panel) strains. The sizes of the different amplicons, as expected for a double crossover event, are indicated by brackets.



Figs. 3.5b-d Agarose gel electrophoretic analysis of the amplification products obtained, following PCR analysis of the *R. solanacearum* wild-type NB336 and mutant NB336Δ*cpaF2* strains using primers CpaF2-F and CpaF2-R (b), CpaF2-R and Gm-R (c) and CpaF2-R and TadB2-F (d). Lanes 1, DNA molecular weight marker; lanes 2, negative control PCR reaction mixture lacking template DNA; lanes 3, genomic DNA from wild-type strain NB336; lanes 4, genomic DNA from mutant strain NB336Δ*cpaF2*. The sizes of the DNA molecular weight marker in base pairs, Hyperladder™ I (Bioline), are indicated to the left of each figure.

To verify the presence of the *lacP-cpaF2* insert DNA, plasmid pLAFR6-LacP-CpaF2 was digested with *Xba*I and either *Hind*III or *Eco*RI. Digestion with *Xba*I and *Eco*RI resulted in the excision of a 1.35-kb DNA fragment corresponding in size to the *cpaF2* gene, whereas digestion of the recombinant plasmid with *Xba*I and *Hind*III yielded two DNA fragments corresponding to the size of the pLAFR-CpaF2 vector DNA (22.350 kb) and the cloned *lacP* promoter (235 bp) (Fig. 3.6b, lanes 6 and 7, respectively). The integrity of the cloned insert DNA was furthermore confirmed by nucleotide sequencing.

The complementation plasmid pLAFR6-LacP-CpaF2 was subsequently introduced into the constructed *R. solanacearum* NB336Δ*cpaF2* mutant strain by electroporation, and a strain that displayed resistance to both gentamycin and tetracycline on TZC agar was selected for further use.

3.3.4 Phenotypic characterization of *R. solanacearum* NB336Δ*cpaF2*

3.3.4.1 Growth curves

It has been noted previously that mutagenesis may influence the growth properties of the mutant strain (Liu *et al.*, 2005). Therefore, it is possible that the observed effects on adherence and virulence, following mutagenesis, may be due to growth impairment of the mutant strain. To investigate whether the introduced mutation influenced the growth properties of the mutant strain, the *R. solanacearum* wild-type NB336 and mutant NB336Δ*cpaF2* strains were cultured in CPG broth, and their growth was followed by taking optical density readings at 600 nm over a time period of 30 h. The results indicated that the mutant NB336Δ*cpaF2* strain displayed a growth rate very similar to the wild-type NB336 strain. Likewise, the mutant NB336Δ*cpaF2* strain complemented with a wild-type copy of the *cpaF2* gene on pLAFR6-LacP-CpaF2 *in trans* also did not display impaired growth (Fig. 3.7).

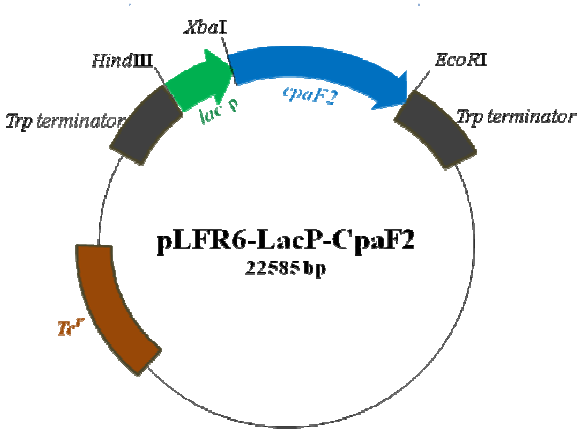


Fig. 3.6a Plasmid map of complementation plasmid pLFR6-LacP-CpaF2.

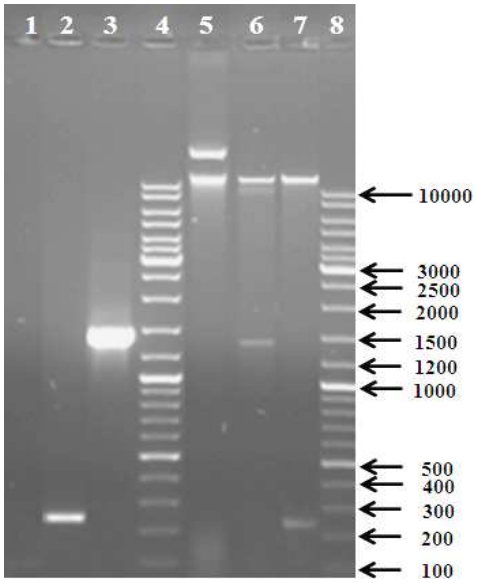


Fig. 3.6b Characterization of complementation plasmid pLAFR6-LacP-CpaF2 by restriction enzyme digestion. Lane 1, negative control PCR reaction mixture lacking template DNA; lane 2 amplicon obtained by PCR amplification using pBluescript SKII (+) plasmid DNA as template and primers LacP-R and LacP-F; lane 3, amplicon obtained by PCR amplification using *R. solanacearum* NB336 genome DNA as template and primers CpaF2-R and CpaF2-F; lanes 4 and 8, DNA molecular weight marker; lane 5, uncut recombinant vector pLAFR6-LacP-CpaF2; lane 6, recombinant vector pLAFR6-LacP-CpaF2 digested with both *Eco*RI and *Xba*I; lane 7, recombinant vector pLAFR6-LacP-CpaF2 digested with both *Xba*I and *Hind*III. The sizes of the DNA molecular weight marker in base bairs, O'GeneRuler™ 100-bp DNA Ladder Plus (Fermentas), are indicated to the right of the figure.

3.3.4.2 Colony and cell morphology

The different *R. solanacearum* strains were investigated with regards to colony morphology on TZC agar medium, which is capable of distinguishing between virulent and non-virulent colony types (Kelman, 1954; Champoiseau *et al.*, 2009). Colonies of the virulent type are irregularly-round, pink or white and fluidal, which contrast with the round, smaller, deep-red and butyrous (dry) non-virulent colonies. Following incubation for 48 h, colonies of the respective *R. solanacearum* strains displayed phenotypes that were in agreement with those described previously for virulent strains, albeit that slight differences were noted. Whereas colonies of the *R. solanacearum* wild-type NB336 strain were fluidal and white with a pale red center, those of the mutant NB336Δ*cpaF2* strain were also fluidal, but were entirely white. Complementation of the mutant strain with the wild-type *cpaF2* gene *in trans* yielded colonies that resembled those of the wild-type *R. solanacearum* NB336 strain (Fig. 3.8a). Cells from stationary phase-cultures of the *R. solanacearum* wild-type, mutant and complemented mutant strains were also examined by scanning electron microscopy (Fig. 3.8b). No noticeable differences in the morphology of the cells from these strains were observed. The respective bacterial cells were short, straight rods and similar in size to each other (1.3±0.3 µm in length and 0.4±0.1 µm in width). These results indicated that disruption the *cpaF2* gene has no apparent effect on colony and cell morphology.

3.3.4.3 Quantification of EPS

Amongst the many virulence factors produced by *R. solanacearum,* it has been reported that exopolysaccharide I (EPS I) is essential for wilting and killing of host plants (Denny, 1995; McGarvey *et al.*, 1998). EPS I is produced in copious amounts both *in vitro* and *in planta* and is believed to function by clogging the xylem vessels, resulting in wilt. EPS I has also been reported to aid in the systemic colonization of susceptible tomato cultivars (Saille *et al.*, 1997; Araud-Razou *et al.*, 1998). Consequently, EPS produced by the *R. solanacearum* wild-type, mutant and complemented mutant strains were quantified following growth in LB broth for 96 h. For this purpose, the concentration of galactosamine was determined as it is a major constituent of the acidic EPS I polymer (Denny *et al.*, 1988; Araud-Razou *et al.*, 1998) and is also considered to be reliable indicator of the total EPS produced (Brumbley and Denny, 1990). The results (Fig. 3.9) indicated that the respective *R. solanacearum* strains produced comparable amounts of EPS (43-45 µg/ml). Based on the results, it was concluded that inactivation of *cpaF2* gene did not affect EPS production in the mutant *R. solanacearum* strain.

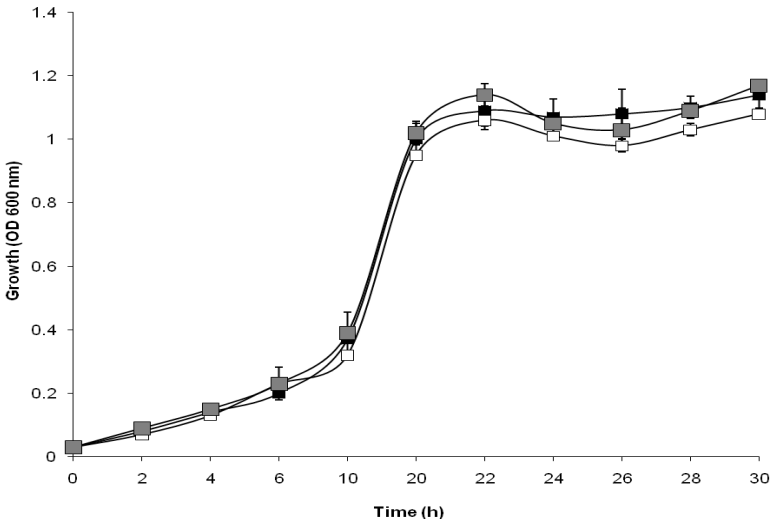


Fig. 3.7 Growth curves of wild-type *R. solanacearum* NB336 (■), mutant NB336Δ*cpaF2* (□) and complemented mutant NB336Δ*cpaF2*::*cpaF2* (▣) strains in CPG broth. The results are the mean of three independent experiments and the error bars represent the standard error of the mean.

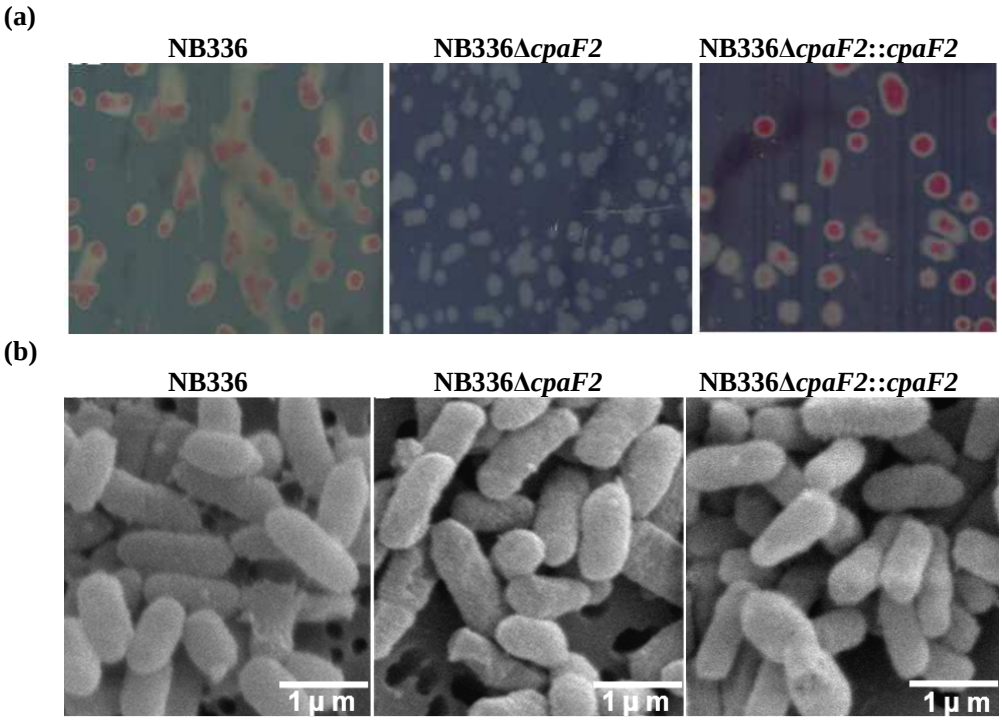


Fig. 3.8 Colony and cell morphology of the *R. solanacearum* wild-type NB336, mutant NB336Δ*cpaF2* and the complemented mutant NB336Δ*cpaF2*::*cpaF2* strains. (a) The respective *R. solanacearum* strains were plated onto TZC agar medium and the colony phenotypes were examined after incubation at 30°C for 48 h. (b) Representative scanning electron microscopy micrographs of cells from stationary phase cultures (24 h) of the respective *R. solanacearum* strains.

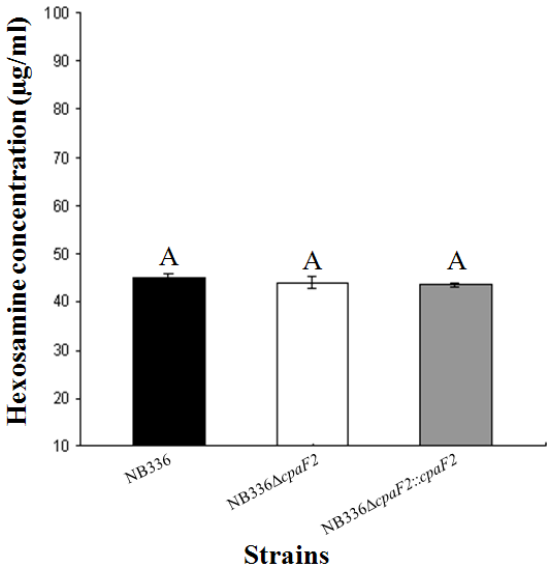


Fig. 3.9 EPS production by *R. solanacearum* wild-type NB336, mutant NB336Δ*cpaF2* and the complemented mutant NB336Δ*cpaF2*::*cpaF2* strains. The respective *R. solanacearum* strains were cultured in LB broth at 30°C for 96 h and the concentration of hexosamine was determined using a modified Elson-Morgan reaction and a galactosamine hydrochloride standard curve. The results are the mean of three independent experiments and the error bars represent the standard error of the mean. Differences in EPS production were not significant at $P = 0.05$.

3.3.4.4 Motility assays

Both swimming motility mediated by flagella and twitching motility mediated by T4ap have been reported to be required for virulence in *R. solanacearum* (Liu *et al.*, 2001; Tans-Kersten *et al*., 2001; Kang *et al.*, 2002). Swimming motility is thought to make its contribution early in disease development by enabling the pathogen to move towards the host rhizosphere, attach to the host roots and invade root tissue (Tans-Kersten *et al*., 2001). Twitching motility has been proposed to be important for colonization of root surfaces, invasion of the root interior, and movement within xylem vessels or migration through other plant tissues (Kang *et al.*, 2002). Due to the important role that motility plays in *R. solanacearum* virulence, it was subsequently investigated whether inactivation of the *cpaF2* gene influenced its motility properties.

To evaluate swimming motility, the wild-type NB336, mutant NB336Δ*cpaF2* and the complemented mutant NB336Δ*cpaF2*::*cpaF2* strains were stab inoculated into motility agar and examined 48 h following inoculation. The colonies of these bacterial strains were surrounded by an even white halo, indicative of flagellum-driven motility (Tans-Kersten *et al.*, 2001). Moreover, the motility halo of the wild-type NB336, mutant NB336Δ*cpaF2* and the complemented mutant NB336Δ*cpaF2*::*cpaF2* strains was similar in size (Fig. 3.10a). To assess twitching motility, the respective bacterial strains were inoculated onto CPG agar and following incubation for 24 h, the colonies were examined under a light microscope. Light microscopy revealed that the colonies of the wild-type, mutant and the complemented mutant strains growing on the surface of the agar medium were indistinguishable from each other. Colonies from the bacterial strains were serrated in appearance, resembling colonies of twitching *R. solanacearum* pictured in the literature (Liu *et al.*, 2001). Also, 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 (Fig. 3.10b). From the above observations, it was thus concluded that the mutant NB336Δ*cpaF2* strain was motile, exhibiting swimming and twitching motility that was comparable to that of the wild-type NB336 strain.

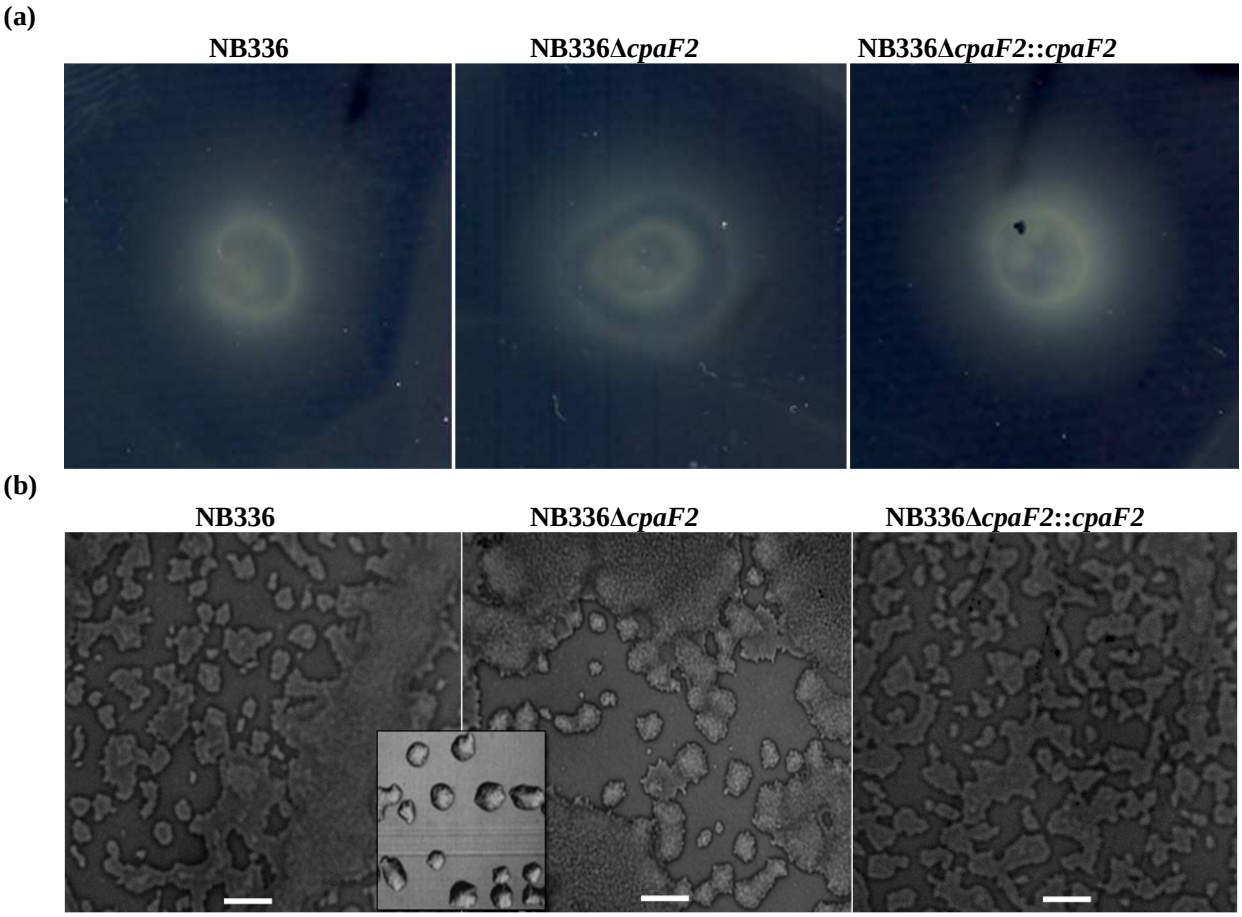


Fig. 3.10 Motility assays of *R. solanacearum* wild-type NB336, mutant NB336Δ*cpaF2* and the complemented mutant NB336Δ*cpaF2*::*cpaF2* strains. (a) For flagellar-mediated swimming motility, the bacterial strains were stab inoculated into motility agar and the zone of colonization was compared after incubation at 30°C for 48 h. (b) For twitching motility, the bacterial strains were cultured on CPG agar and examined 20-24 post-inoculation under a light microscope. For the wild-type, mutant and the complemented mutant strains, motile rafts could be seen, indicative of twitching motility across the agar surface. A *pilQ* mutant of *R. solanacearum* that does not display twitching motility (from Liu *et al.*, 2001) is shown in the inset for comparative purposes. Bar = 10 µm.

3.3.4.5 Biofilm development

Many plant-associated bacteria form biofilms in contact with biotic or abiotic environments (O'Toole and Kolter, 1998; Dow *et al*., 2003; Koutsoudis *et al*., 2006; Danhorn and Fuqua, 2007). 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; Kao *et al.*, 1992), 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). Having established that the mutant *R. solanacearum* NB336Δ*cpaF2* strain was capable of swimming and twitching motility, indicating normal production of flagella and T4ap, it was next investigated whether the mutant strain was capable of forming biofilms. For this purpose, a quantitative microtiter plate assays was used, as described in Materials and Methods (Section 3.2.10.5).

The results indicated that although each of the *R. solanaceraum* strains formed biofilm bands at the air-liquid interface, differences were noted in the amount of biofilm formed. Interestingly, the mutant NB336Δ*cpaF2* strain consistently formed significantly more biofilm than the wild-type NB336 strain. However, strain NB336Δ*cpaF2*::*cpaF2*, in which the mutation was complemented *in trans*, produced a similar amount of biofilm compared to the wild-type NB336 strain (Fig. 3.11). These results indicated that the mutant *R. solanaceraum* strain had significantly higher biofilm formation ability on an abiotic surface than the wild-type under tested conditions.

3.3.5 Plant assays

3.3.5.1 *In planta* growth curve

Previously, it was shown that the *R. solanacearum* wild-type NB336 and mutant NB336Δ*cpaF2* strains displayed similar growth rates in CPG broth (Fig. 3.7). To determine whether these bacterial strains also displayed similar growth properties *in planta*, the respective bacterial strains were inoculated into the stems of potato plants and bacterial counts

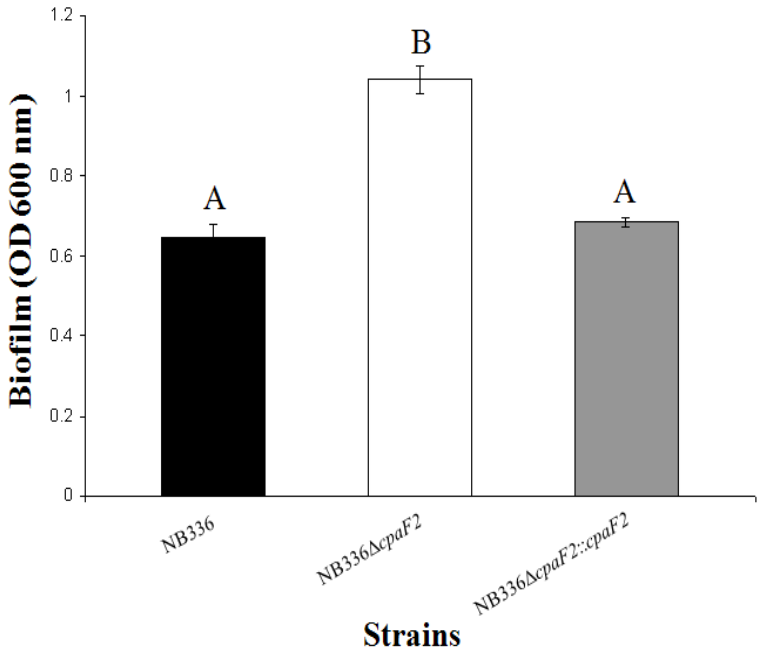


Fig. 3.11 Biofilm formation by the *R. solanacearum* wild-type NB336, mutant NB336Δ*cpaF2* and the complemented mutant NB336Δ*cpaF2*::*cpaF2* strains. Biofilm formation was quantified by measuring the OD_{600} of crystal violet-stained PVC microtitre wells. The results are the mean of three independent 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.

were performed at 10-h intervals over a time period of 50 h. The results indicated that there was no significant difference in the growth rate between the wild-type and mutant strains *in planta* (Fig. 3.12).

3.3.5.2 Potato root colonization assay

The ability to adhere to plant roots is an important early step for *R. solanacearum* to cause disease in susceptible plants (Vasse *et al*., 1995; 2005). Adhering pathogens typically have one or more extracellular molecules (*e.g.* carbohydrate molecules and non-pilus adhesins), or surface appendages (*e.g.* T4a pili), that interact with various surfaces (Danhorn and Fuqua, 2007). In the case of *R. solanacearum*, both flagella and T4a pili have been implicated in the adherence of the bacteria to host cells (Kang *et al.*, 2002; Tans-Kersten *et al.*, 2004). Although other types of pili, such as Flp pili, have been implicated in adherence and virulence of pathogens infecting humans (Kachlany *et al.*, 2000; Spinola *et al.*, 2003; de Bentzmann *et al.*, 2006), it is not known whether they may have a similar function in phytopathogens.

To assess adherence to and colonization of potato roots, potato plants were inoculated with the *R. solanacearum* wild-type NB336, mutant NB336Δ*cpaF2* and complemented mutant NB336Δ*cpaF2*::*cpaF2* strains using a soil soak inoculation method. After 15 days, the extent of root colonization was assessed by scanning electron microscopy (SEM) of the plant roots and by enumeration of viable cells recovered from the roots. Visualization of the roots by SEM indicated that cells of both the wild-type and complemented mutant strains were more abundantly present on the surface of the roots compared to cells of the mutant strain (Fig. 3.13a). In support of this qualitative data, quantitative data obtained by viable cell-plating indicated that significantly less bacteria were recovered from roots of plants inoculated with the mutant NB336Δ*cpaF2* strain than those inoculated with the wild-type and complemented mutant strains (Fig. 3.13b). Although these results therefore indicate that the *R. solanacearum* wild-type NB336 and mutant NB336Δ*cpaF2* strains differ in their ability to adhere to and/or colonize plant roots, it does not exclude the possibility that this difference may be due to a difference in the survival of the mutant strain in the soil.

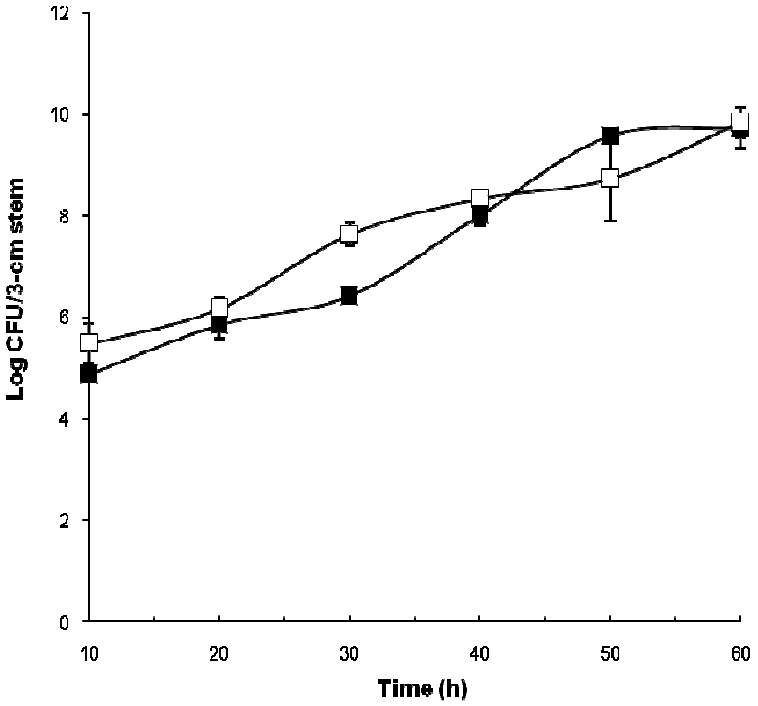


Fig. 3.12 *In planta* growth curves of *R. solanacearum* wild-type NB336 (■) and mutant NB336Δ*cpaF2* (□) strains. The results are the mean of three independent experiments and the error bars represent the standard error of the mean.

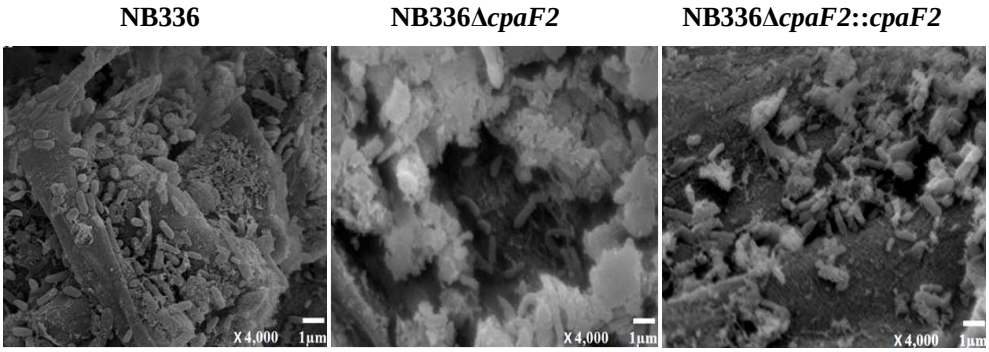
The soil survival of the *R. solanacearum* wild-type NB336, mutant NB336Δ*cpaF2* and complemented mutant NB336Δ*cpaF2*::*cpaF2* strains was subsequently investigated in potting soil without plants by incubating beakers of inoculated soil under greenhouse conditions. The number of viable cells that could be recovered from the potting soil was determined up to 15 days after inoculation. Over this period, there were less than 10-fold changes from the initial inoculum concentration, to *ca.* 4.8×10^5 CFU/g of soil being recovered on the last sampling date for each of the strains tested. However, none of the strains were significantly different from each other at a given sampling date (Fig. 3.14). These results therefore showed that all strains were equally available for colonization within the first 15 days after soil inoculation, which is past the time at which disease symptoms developed in plants inoculated with the wild-type mutant NB336 strain (see Fig. 3.16). Cumulatively, these results therefore suggest that the poor colonization of potato roots by the mutant NB336Δ*cpaF2* strain is not due to an inability to survive in the soil, but rather due to attenuated adherence to the plant roots.

3.3.5.3 Virulence assays

Prior to undertaking virulence assays, the *R. solanacearum* wild-type NB336, mutant NB336Δ*cpaF2* and complemented mutant strains were evaluated for their ability to elicit a HR defense response when infiltrated into non-host tobacco leafs. Like the wild-type strain, both the mutant and complemented mutant strains produced necrotic lesions in the leaves of the indicator plant that were similar to that elicited by the positive control phytopathogen, *Xanthomonas campestris* (Fig. 3.15). Based on the ability of the mutant *R. solanacearum* NB336Δ*cpaF2* strain to elicit a normal HR defense response, it was thus concluded that the T3S system, which is responsible for the secretion of the majority of virulence factors, was not affected by the introduction of the *cpaF2*::*Gm*^{r} mutant allele.

To determine the involvement of the megaplasmid *tad* locus in wilt disease development, two types of virulence assays were performed on a susceptible potato host. In the naturalistic soil soak assay, which requires bacteria to locate and invade host roots from the soil (Vasse *et al.*, 1995; 2005), the wild-type strain NB336 caused a disease index of 3.94 at 16 days after inoculation and 4 at 30 days after inoculation. In contrast, the mutant NB336Δ*cpaF2* strain did not cause disease over the 30-day period. Notably, the complemented mutant strain NB336Δ*cpaF2*::*cpaF2* restored the virulence phenoptype. Although the complemented strain caused a disease index of 4 at 30 days, it was significantly (P = 0.05) slower than the

(a)



(b)

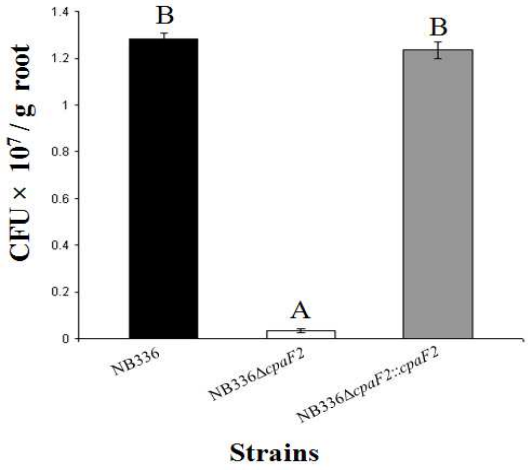


Fig. 3.13 Colonization of potato plant roots by the *R. solanacearum* wild-type NB336, mutant NB336Δ*cpaF2* and the complemented mutant NB336Δ*cpaF2*::*cpaF2* strains. Potato plants were inoculated by soil soaking with standardized cultures of the respective *R. solanacearum* strains. After 15 days, the potato plants were removed, the roots were rinsed with sterile dH_2O, blotted dry on absorbent paper, excised and either examined under a scanning electron microscope (SEM) or ground, diluted serially and plated onto TZC agar medium. (a) Representative SEM micrographs of potato roots showing colonization of the roots by the *R. solanacearum* wild-type NB336, mutant NB336Δ*cpaF2* and the complemented mutant NB336Δ*cpaF2*::*cpaF2* strains. (b) Number of bacterial CFU recovered from potato plant roots. 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.

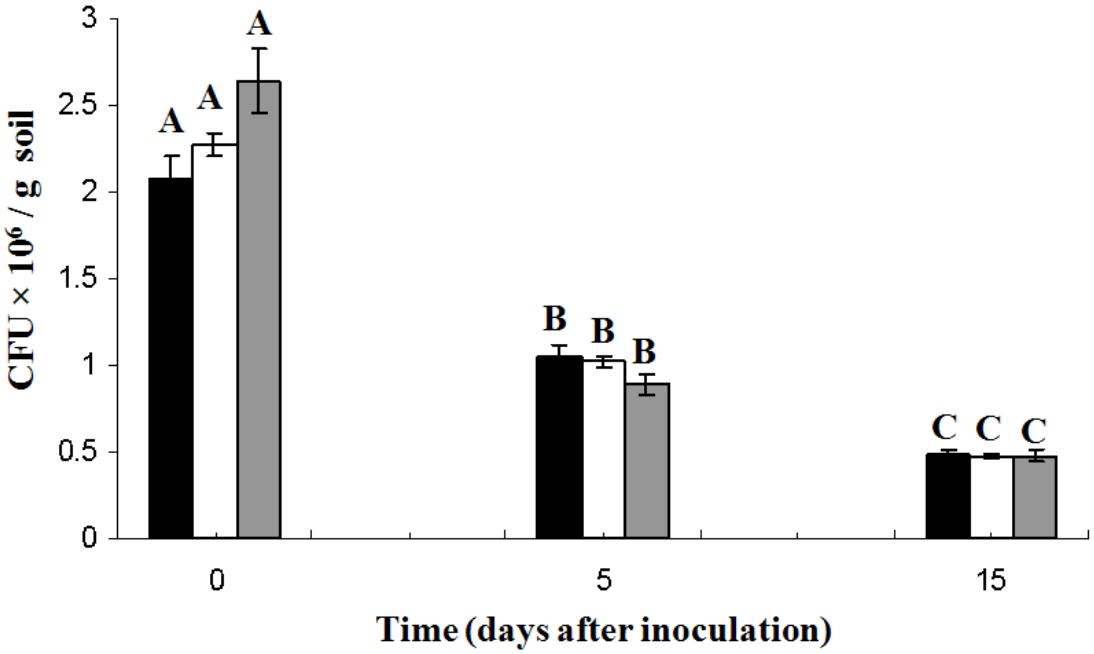


Fig. 3.14 Survival of *R. solanacearum* wild-type NB336 (■), mutant NB336Δ*cpaF2* (□) and complemented mutant NB336Δ*cpaF2*::*cpaF2* (▩) strains in soil. Soil was inoculated with standardized cultures of the respective *R. solanacearum* strains and the number of bacterial CFU recovered at different days after inoculation was determined by dilution plating onto TZC agar medium. 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) in bacterial CFU at different times after inoculation.

wild-type strain in causing wilt symptoms (Fig. 3.16a). In a cut-petiole assay, which bypasses the normal infection route and introduces bacteria directly into the vascular system (Saille *et al*., 1997; Liu *et al*., 2001), the wild-type NB336 strain wilted all potato plants by day 10 after inoculation. The complemented mutant strain NB336Δ*cpaF2*::*cpaF2* wilted all potato plants by day 16 and was statistically indistinguishable from the wild-type strain in this assay. However, as with the soil soak assay, potato plants inoculated with the mutant NB336Δ*cpaF2* strain did not wilt and hardly developed disease symptoms, except for restricted leaf yellowing and localized necrosis on some inoculated potato plants (Fig. 3.16b). These results therefore suggested that the megaplasmid *tad* locus of *R. solanacearum*, or functions that require the *tad*-encoded proteins, is important for virulence on potato plants.

3.4 DISCUSSION

Bacteria can assemble various appendages on the cell surface that allows them to colonize diverse biotic and abiotic surfaces. Studies on *R. solanacearum* have revealed that flagella and T4ap are involved in processes such as motility, attachment or both (Tans-Kersten *et al.*, 2001; Liu *et al.*, 2001; Kang *et al.*, 2002). In addition to these cell surface appendages, *R. solanacearum* also harbors two *tad* loci whose genes encode components previously reported in *A. actinomycetemcomitans* (Kachlany *et al.*, 2000; 2001) and *P. aeruginosa* (de Bentzmann *et al.*, 2006) to be involved in the assembly of Flp pili that support adherence. 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, the relevance of the *tad* gene products in *R. solanacearum* adherence and virulence on potato plants was investigated.

Although *R. solanacearum* harbors two *tad* loci, they differ in their genetic composition (Chapter 2, Fig. 2.1). In contrast to the *tad* locus in the megaplasmid, the chromosomal *tad* locus lacks a discernible homologue of the TadD protein, which is required for the proper assembly and/or function of the secretin complex (Clock *et al.*, 2008). Specifically, TadD is essential for RcpA secretin abundance and RcpA does not multimerize or localize to the outer membrane without expression of TadD. Moreover, during *in vivo* assays to identify virulence

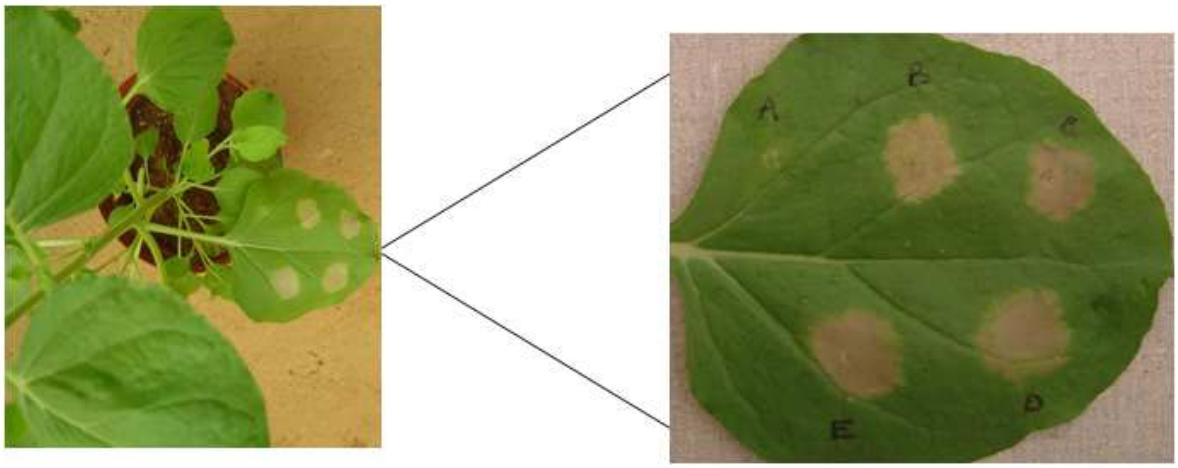
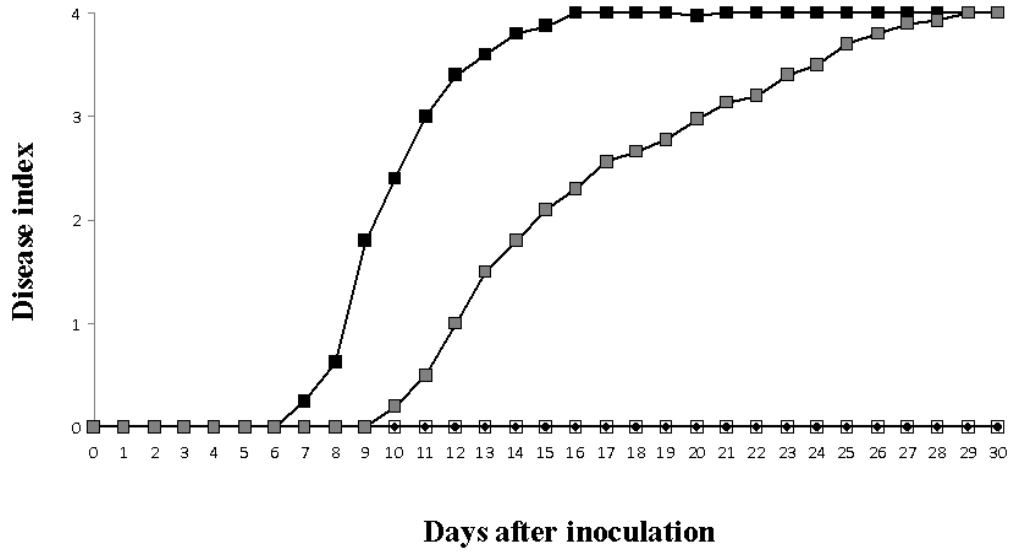


Fig. 3.15 Hypersensitivity reaction (HR)-dependent lesion formation in a tobacco plant inoculated with different *R. solanacearum* strains. The tobacco plant leaf was infiltrated with standardized suspensions of the *R. solanacearum* wild-type NB336 (C), mutant NB336Δ*cpaF2* (D) and complemented mutant NB336Δ*cpaF2*::*cpaF2* (E) strains. As controls, the leaf was also infiltrated with sterile dH_2O (A) and *Xanthomonas campestris* (B). The photographs show lesions after 7 days of incubation. An enlarged view of the infiltrated leaf and associated lesions is presented to the right.

(a)



(b)

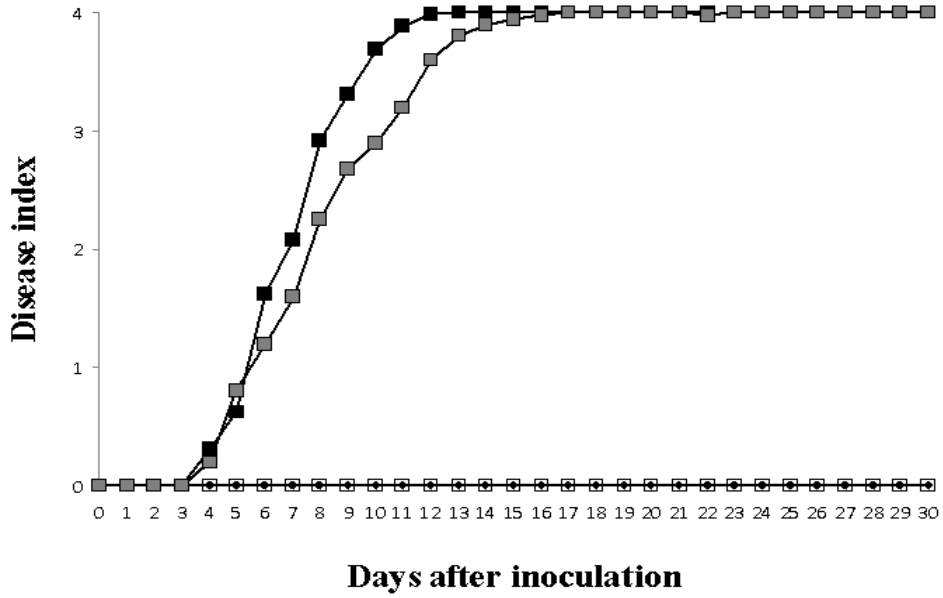


Fig. 3.16 Disease progress of the *R. solanacearum* wild-type NB336 (■), mutant NB336ΔcpaF2 (□) and complemented mutant NB336Δ*cpaF2*::*cpaF2* (■) strains on potato plants by different inoculation methods. Thirty-day old potato plants were inoculated either by soaking the soil (a) or by applying the 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 different from that of the mutant NB336ΔcpaF2 strain (P = 0.05). In (b), the virulence of the wild-type and complemented mutant strains was not significantly different, but both differed from that of the mutant NB336ΔcpaF2 strain (P = 0.05).

genes, *tadD* was identified as likely to be critical for virulence in the animal pathogens *Pasteurella multocida* and *Yersinia rukeri* (Fuller *et al.*, 2000; Fernandez *et al.*, 2004). Since the megaplasmid *tad* locus of *R. solanacearum* contains a TadD homologue (RSP1082) and considering that most of the virulence genes of this bacterium are located in the megaplasmid (Salanoubat *et al.*, 2002; Genin and Boucher, 2004), this *tad* locus was therefore selected for mutagenesis. A targeted mutation was introduced into the *cpaF2* gene, which is a homologue of the *tadA* gene that encodes an NTPase required for energizing the assembly or secretion of Flp pili in *A. actinomycetemcomitans* (Bhattarcharjee *et al.*, 2001). Notably, a *tadA* mutant of the human pathogen *H. ducreyi* is avirulent (Nika *et al.*, 2002) and it has also been reported that *tadA* mutants of several 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). The mutant *R. solanacearum* strain generated was designated NB336Δ*cpaF2* and characterized by Southern blot analysis and PCR analyses, the results of which confirmed that the NB336Δ*cpaF2* strain contains a single interrupted copy of *cpaF2*. To ensure that the derived mutant strain is not polar, the *cpaF2* mutation was genetically complemented with the wild-type *cpaF2* gene *in trans*. Near complete complementation of all phenotypes investigated was observed, showing that the insertion in the mutant NB336Δ*cpaF2* strain is indeed non-polar.

In this study, Flp pili encoded by *tad* gene clusters could not be detected on the surface of *R. solanacearum* strain NB336 by transmission electron microscopy (TEM) (results not shown). Similar observations were also previously made for the human pathogens *H. ducreyi* (Nika *et al.*, 2002) and *Yersinia enterocolitica* (Schilling *et al.*, 2010), whereas the Flp pili of *P. aeruginosa* are only visible by TEM on the cell surface if the *flp* gene is overexpressed from a plasmid (de Bentzmann *et al.*, 2006). However, the literature regarding pili in *R. solanacearum* suggests that this bacterium can make different 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 polar pili. The HrpY pilus is composed of pilin monomers (7 kDa) encoded by the *hrpY* gene, whereas the T4a pilus is composed of pilin monomers (17 kDa) encoded by the *pilA* gene. The role of these pili in adherence and/or virulence of *R. solanacearum* have been investigated. It was shown that although HrpY pili are an essential component of the T3S system, they are not required for adherence as a *hrpY* mutant of *R. solanacearum* GMI1000 adhered to both host and non-host plant cells grown in suspension culture (Aldon *et al.*, 2000; van Gijsegem *et al.*, 2000). The T4ap was shown to be required for twitching motility by *R. solanacearum*, which promoted

virulence on tomato plants (Kang *et al.*, 2002). Similar studies on other vascular pathogens, such as *Xylella fastidosa* (Li *et al.*, 2007; Meng *et al.*, 2005) and *Acidovorax avenae* subsp. *citrulli* (Bahar *et al.*, 2009), have confirmed the importance of T4ap for twitching motility and indicated that this activity contributes to spread of these pathogens via the xylem vessels. In addition to the above reports, two different groups (Young *et al.*, 1985; Stemmer and Sequiera, 1987) reported purified pilin protein from *R. solanacearum* strain K60 of which neither the molecular mass (9.5 kDa) nor the amino acid compositions matched either HrpY or T4a pilins. Although it is likely that both these groups isolated the same pilin, the results do, however, indicate the presence of at least a third distinct type of pilus in *R. solanacearum*. In support of this statement, 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 T4ap was reported to have no quantitative effect on adherence to host or non-host cells (Kang *et al.*, 2002), thus suggesting the presence of an as yet uncharacterized attachment factor.

Based on the genetic evidence presented in this study, the results indicated that both the mutant NB336Δ*cpaF2* and wild-type NB336 *R. solanacearum* strains had a mucoid colony morphology on solid medium, displayed similar motility, had similar growth *in vitro* and *in planta* under tested conditions, and they produced similar amounts of EPS. Moreover, like the wild-type NB336 strain, the mutant NB336Δ*cpaF2* strain elicited a normal HR response when infiltrated into non-host tobacco plants, indicating the presence of HrpY pili and that the T3S system also was not affected. The wild-type and mutant strains, however, differed from each other with regards to biofilm formation, potato root colonization and virulence. This therefore suggests that a functional *cpaF2* gene (and presumably Flp pili) plays an important role at some point during disease development.

Biofilm formation has been associated with virulence of pathogenic bacteria. In the case of phytopathogenic bacteria, biofilms are thought to contribute to virulence through several mechanisms, including enhanced localization of specific niches, increased resistance to antimicrobials released by the plant and/or blockage of sap flow in the xylem vessels (Meng *et al.*, 2005; Danhorn and Fuqua, 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 *R. solanacearum* mutant NB336Δ*cpaF2* overproduced biofilms compared to the wild-type NBB336 strain (Fig. 3.11). 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). However, the result is in agreement with that reported for the human pathogen *Y. enterocolitica* (Schilling *et al.*, 2010), indicating that Flp pili are likely not required by *R. solanacearum* for biofilm formation on an abiotic surface. Indeed, in the case of *R. solanacearum*, the presence of a functional *cpaF2* gene appears to mask the contribution of other cell surface appendages, such as the T4ap, to the biofilm formation process. This suggests that T4ap, but not Flp pili encoded by the *tad* gene cluster, is important for biofilm formation by *R. solanacearum*. This statement is supported by the fact that T4ap 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.

R. solanacearum enters a plant through the roots, penetrates the xylem, systemically colonizes the stem and causes wilt symptoms (Vasse *et al.*, 1995; 2005). Mutation of the *R. solanacearum cpaF2* gene resulted in a mutant that did not display disease development on susceptible potato plants in a biologically representative soil soak inoculation assay. Experiments designed to determine whether reduction in virulence in this assay was due to poor survival in soil or due to reduced adherence and colonization of potato roots indicated no difference in soil survival between the mutant NB336Δ*cpaF2* and wild-type NB336 strains (Fig. 3.14). However, a significant reduction in adherence to and colonization of the potato roots was observed, both qualitatively by SEM and quantitatively by viable plate counts. Compared to the wild-type NB336 and complemented mutant strains, which both formed cell clumps resembling a biofilm on potato roots, the cells of the mutant NB336Δ*cpaF2* strain adhered only as sparse isolated bacteria (Fig. 3.13). The non-virulence phenotype of the mutant strain NB336Δ*cpaF2* could not be rescued by cut-petiole inoculations. In contrast, the wild-type and complemented mutant strains were able to cause disease, resulting in wilting and death of the potato plants. These results therefore suggest that *cpaF2* (and presumably Flp pili) make their contribution to virulence before bacteria reach the vascular system. Thus, it may be required during the very early steps of infection, perhaps by facilitating the initial attachment of the bacterium to potato plant tissue. Compared to the results obtained regarding biofilm formation on an abiotic surface, these results furthermore suggests that Flp pili may preferentially mediate adherence to plant tissue. In this regard it is interesting to note that the Flp pilin of both *A. actinomycetemcomitans* and *P. aeruginosa* is glycosylated (Tomich *et al.*, 2006; Bernard *et al.*, 2009). Although there does not appear to be a clear role for glucans in adherence (Marceau *et al.*, 1998), it is tempting to speculate that modified Flp pilin may

promote binding to plant cell receptors. However, this is tenuous at present and requires further mutagenesis study.

Based on results obtained in this study, it can be hypothesized that different *R. solanacearum* cell surface appendages act cooperatively to cause wilt disease, yet have distinct roles in this process. Flagella enable the pathogen to move through the soil towards the host rhizosphere (Tans-Kersten *et al.*, 2004), whereas Flp pili are required for initial attachment to the plant roots (this study). The importance of T4ap for successful infection of the host plant may be beyond the stage of adhesion by the bacterium. The T4ap may contribute to cell-to-cell contact and promote twitching motility to allow for increased colonization of the root surface, as well as access to and migration through the xylem vessels (Kang *et al.*, 2002). It therefore follows that if any of these processes are impaired, then disease development is diminished or does not ensue.

In conclusion, the results of this study provide strong support that the *tad* locus in the megaplasmid genome of *R. solanacearum* is essential for adherence and expression of virulence on potato plants. Conversely, the chromosomal *tad* locus of *R. solanacearum* does not appear to play a role in the phenotypes investigated. Why *R. solanacearum* maintains these two *tad* loci is not clear. However, it may be that the chromosomal *tad* locus carries no longer or not yet used genes that might serve as a surplus material for further developments, an advantage that may compensate for the cost of maintaining these genes during evolution. Nevertheless, the identification and study of genes involved in the initial stages of pathogenicity can be invaluable in the development of effective preventative control measures for *R. solanacearum*. The continued study of the *tad* loci of this bacterium may shed some light on factors that determine successful colonization of a plant by *R. solanacearum* and perhaps open up new avenues of disease control.

CHAPTER FOUR

CONCLUDING REMARKS

Ralstonia solanacearum is a soil-borne pathogen that causes bacterial wilt of diverse plant species, including economically important plants such as tomato, peanut and potato (Janse *et al.*, 2004; Swanson *et al.*, 2005). Due to its wide geographic distribution and unusually broad host range, the pathogen is responsible for severe crop losses worldwide (Swanson *et al.*, 2005). Many factors that contribute to bacterial wilt disease have been identified, which include several plant cell wall-degrading enzymes, extracellular polysaccharide (EPS) and some type III-secreted effectors (McGarvey *et al.*, 1999; Gonzalez and Allen, 2003; Genin *et al.*, 2005). It has also been demonstrated that cell surface appendages, such as flagella and type IVa pili (T4ap), are involved in the pathogenicity of *R. solanacearum* (Tans-Kersten *et al.*, 2001; Liu *et al.*, 2001; Kang *et al.*, 2002). In addition to T4ap, several reports have implicated a distinct subfamily of type IVb pili (T4bp) in the colonization and virulence of different human and animal pathogenic bacteria (Kachlany *et al.*, 2000; Fuller *et al.*, 2000; Nika *et al.*, 2002; Fernandez *et al.*, 2004; de Bentzmann *et al.*, 2006). These pili, designated Flp pili, are assembled by a dedicated machinery that is encoded by the *tad* gene cluster (Kachlany *et al.*, 2000; Nika *et al.*, 2002; de Bentzmann *et al.*, 2006), and is found in a wide variety of bacteria and archaeal species (Planet *et al.*, 2003; Tomich *et al.*, 2007). Consequently, the aim of this investigation was essentially to determine the importance of the *tad* locus in *R. solanacearum* virulence on potato. The details of the results obtained in the course of achieving this objective have been discussed in the individual Chapters. The new information that has evolved during this investigation will be summarized briefly and suggestions regarding future research will be made.

In this study the genome sequence of the *R. solanacearum* tomato pathovar GMI1000 (Salanoubat *et al.*, 2002) was mined to identify genes involved in the assembly of Flp pili. Two distinct 14-gene clusters were identified whose gene products encoded components previously reported as being involved in the assembly of Flp pili. The respective gene clusters were located in the chromosome and megaplasmid of *R. solanacearum* (Chapter 2). The genes in both loci are organized linearly in a single direction that suggests that they may constitute an operon. This is likely, as the *tad* locus of both *Haemophilus ducreyi* (Nika *et al.*, 2002) and *Aggregatibacter actinomycetemcomitans* (Haase *et al.*, 2003) is transcribed as a polycystronic mRNA. Both of the *R. solanacearum tad* gene clusters are preceded by sigma 70 (σ^{70})- and sigma 54 (σ^{54})-dependent promoter regions. In this regard, it is interesting to note that protein RSP1079, encoded by the megaplasmid *tad* locus only, displays amino acid

sequence identity with the transcriptional regulator PilR (Darzins and Russel, 1997). Although a histidine sensor kinase could not be identified, it does not exclude the possibility that RSP1079 may display activity independent of a sensor kinase as reported previously for various other two-component regulators (Ma *et al.*, 1998; Schär *et al.*, 2005; Kehl-Fie *et al.*, 2009; Bernard *et al.*, 2009). Based on its sequence identity with PilR and the presence of a σ^{54}-dependent promoter sequence, which requires and enhancer binding protein to allow for expression (Buck *et al.*, 2000; Stock *et al.*, 2000), it is tempting to suggest that RSP1079 might be such a response regulator that positively controls expression of the megaplasmid *tad* gene cluster. Future experiments, involving transcriptional fusions of the different promoter regions may aid in determining the involvement of these promoter regions in expression of the *tad* gene clusters, whilst a transcriptional fusion of the σ^{54}-dependent promoter region and a mutant strain lacking RSP1079 might yield some insight into the regulation of this megaplasmid *tad* locus promoter region. These constructs may also be used towards identifying environmental conditions that influence *R. solanacearum tad* gene expression. Indeed, environment-dependent modulation of these gene clusters appears to be a common feature, having been reported in *A. actinomycetemcomitans* (Tomich *et al.*, 2007), *Caulobacter crescentus* (Viollier *et al.*, 2002) and *Pseudomonas aeruginosa* (Bernard *et al.*, 2009). In addition to studies aimed at understanding regulatory aspects of *R. solanacearum tad* gene expression, studies also aimed at elucidating the structure and function of the encoded proteins will undoubtedly help to define the mechanism by which these *R. solanacearum* systems operate.

To investigate the importance of the *tad* loci in the ability of *R. solanacearum* to adhere to and cause disease on potato plants, a mutant *R. solanacearum* NB336 strain (potato pathovar) was constructed by inserting a gentamycin gene cassette into the *cpaF2* (RSP1085) gene of the megaplasmid *tad* locus. This was based, amongst other, on the observations that the megaplasmid *tad* locus harbors most of the *R. solanacearum* virulence genes (Salanoubat *et al.*, 2002; Genin and Boucher, 2004), and that CpaF2 is a homologue of TadA, which serves as the energy modulator for secretion and assembly of the Flp pili (Bhattacharjee *et al.*, 2001). Moreover, previous studies on different human pathogenic bacteria have indicated that *tadA* mutants of these bacteria lack Flp pili on the cell surface (Bhattacharjee *et al.*, 2001; Nika *et al.*, 2002; de Bentzmann *et al.*, 2006). Characterization of the non-polar mutant strain NB336Δ*cpaF2* indicated that it had a mucoid colony morphology on solid medium, grew as well as the wild-type strain in medium and *in planta* under tested conditions, showed normal

swimming and twitching motility, elicited a normal hypersensitivity response, and was not affected in its ability to produce EPS. However, in two different virulence assays, the mutant NB336Δ*cpaF2* strain was avirulent, indicating that CpaF2 (and presumably Flp pili) is important for properties such as adherence to potato plant roots and virulence (Chapter 3). In this study, it was not possible to demonstrate physical presence of the Flp pili. The inability to visualize these cell surface appendages by transmission electron microscopy (TEM) may be due to the amount of pili expressed being too low to be detected or the Flp pili may have disassembled during processing for electron microscopy. Nevertheless, based on the genetic evidence presented, the results suggested that the reduction in virulence was due to a decline in root adhesion and thus indicated that CpaF2 makes its contribution to pathogenesis at the stage of initial attachment to plant tissue.

Early attachment events, such as those described above, could in future be studied by tagging the wild-type and mutant *R. solanacearum* strains with a green fluorescent protein (GFP) and observing early attachment interactions between the fluorescent *R. solanacearum* cells and potato plant roots under a confocal laser scanning microscope. These types of studies may also provide insights into the contribution, if any, that Flp pili make to polar adherence of this bacterium. It has been reported that *R. solanacearum* displays polar adhesion to suspension-cultured cells which might be important in the ability of the type III secretion (T3S) system to deliver effector proteins that promote pathogenesis (van Gijsegem *et al.*, 2000; Liu *et al.*, 2001; Kang *et al.*, 2002). Although the location of Flp pili in *R. solanacearum* is not yet known, it is likely to be polar. This is based on reports indicating that the Flp pili of *C. crescentus, P. aeruginosa* and *A. actinomycetemcomitans* are polar (Skerker and Shapiro, 2000; de Bentzmann *et al.*, 2006; Tomich *et al.*, 2007). Moreover, the *tad l*oci of *R. solanacearum* encode a putative TadZ/CpaE protein that, in the case of *C. crescentus*, has been shown to be responsible for localizing the Flp biogenesis and secretion machinery to the cell pole (Viollier *et al.*, 2002).

In contrast to *P. aeruginosa*, where the T4bp adherence role is overcome by the presence of flagella and T4ap (de Bentzmann *et al*., 2006), in *R. solanacearum*, T4a and T4b pili appear to have distinct roles. Whereas T4ap mediate adherence to abiotic surfaces and twitching motility, the T4bp mediate adherence to biotic surfaces and virulence. This is similar to observations made by Meng *et al*. (2005), in which type I and T4ap appear to play distinct roles in biofilm formation and twitching motility of *Xylella fastidiosa*, respectively. A *R.*

solanacearum T4ap mutant, which is twitching- and biofilm-deficient, was reported to be still able to adhere to tobacco tissues and cause wilting disease on tomato plants (Kang *et al.*, 2002). Interestingly, the results obtained during the course of this investigation suggest that the Flp pili, a T4bp of *R. solanacearum* may confer some specificity in the interaction between the bacterium and plant tissue. This was evidenced by the drastically reduced adherence of the mutant NB336Δ*cpaF2* strain to potato plant roots (Fig. 3.13). In contrast, the mutant strain formed a copious amount of biofilm on an abiotic plastic surface (Fig. 3.11). The overproduction of biofilm by the mutant strain compared to the wild-type strain therefore suggests that the Flp pili may be of lesser importance in adherence to an abiotic surface and that this attachment event is likely mediated by the T4ap. These results are reminiscent of those obtained for *P. aeruginosa* in which an *rcpC* mutation had no effect on biofilm formation but decreased adhesion to epithelial cells (Bernard *et al.*, 2009). It has been reported that Flp pili of *A. actinomycetemcomitans* and *P. aeruginosa* are posttranslationally modified, most likely by glycosylation, 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). It would therefore be of interest to determine whether the difference in the observed adhesion phenotypes of *R. solanacearum* is linked to Flp pilin modification. Consequently, mutagenesis studies should be performed to determine if the Flp pilin of *R. solanacearum* is likewise modified and whether this modification plays a role in the apparent preference of the Flp pili to mediate adherence biotic surfaces.

Although *tad* loci have been identified in various bacterial species, usually as a single copy, sequence analyses have identified up to three copies in some species (Tomich *et al.*, 2007). The functional significance of bacteria harboring multiple copies of the *tad* locus has not yet been investigated. Nevertheless, the results obtained in this study suggest that the chromosomal *tad* locus is dispensable for the adherence and virulence traits of *R. solanacearum* NB336, since the phenotypes were restored only if a wild-type copy of the *cpaF2* gene was provided *in trans* to the mutant NB336Δ*cpaF2* strain. It should be noted that although the genome sequence of *R. solanacearum* NB336 is not known, it is likely that this strain, like strain GMI1000, also harbors two *tad* loci. Sequence analysis of amplicons amplified from the wild-type NB336 strain (Fig. 3.4a) indicated that these amplicons were similar to the *cpaF1* and *cpaF2* genome regions of *R. solanacearum* GMI1000. However, the results do not exclude the possibilities that the chromosomal *tad* gene cluster is either not expressed under the conditions used in this study, or that it may be expressed but is unable to

assemble into a functional Flp biogenesis and secretion apparatus. With regards to the latter, it is tempting to speculate that the absence of a *tadD* homologue in the *R. solanacearum* chromosomal *tad* locus may prevent the proper assembly of the RcpA secretin of the Flp biogenesis apparatus. This is not unlikely as TadD, a putative lipoprotein that is localized to the bacterial outer membrane, has been reported to be critical for the assembly, transport and/or function of the RcpA secretin (Clock *et al.*, 2008). It is therefore conceivable that the inability to assemble a proper secretin complex may prohibit the assembly and/or secretion of the Flp pilus.

In conclusion, this investigation demonstrates that *R. solanacearum* requires a megaplasmid *tad* locus, for adherence and 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. The challenge is now to determine more precisely how the *R. solanacearum tad* gene products promote attachment and what their specific roles are in the pathogenesis of bacterial wilt. The continued study of these gene clusters will not only enable a better understanding of this apparently important virulence factor, but also raises the possibility of developing new control strategies against this devastating phytopathogen.

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