Genomic and functional characterization of motility in *Pantoea ananatis*

by

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DECLARATION

I, Tania Weller-Stuart, declare that the thesis, which I hereby submit for the degree Philosophiae Doctor 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.

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Tania Weller-Stuart

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PREFACE

Pantoea ananatis causes disease symptoms in a wide range of economically important plants such as *Eucalyptus*, maize and onions. This study specifically focussed on the interactions of *P. ananatis* LMG20103 with onion seedlings where it causes symptoms that include water-soaked lesions, wilting and bleaching of the leaves, and maceration of the bulbs. The pathogenicity of *P. ananatis* is not well understood, however, motility plays an important role in many other well-known phytopathogens such as *Ralstonia solanacearum* and *Pseudomonas syringae*. It is therefore hypothesised that *P. ananatis* uses motility to colonise and infect its hosts.

Chapter 1 reviews the literature on how motility aids phytopathogenic bacteria in locating their host, attaching and initiating infection, as well as dissemination. The two dominant forms of motility utilised are swimming and twitching motility. Swimming motility is essentially the rotation of flagella which propels the bacterial cell forward through a fluid environment in response to chemotactic signals. The motor that drives the flagella is made up of several proteins that include the MotAB proteins and its function is dependent on a proton motive force. While twitching motility is not as fast as swimming motility, it is a rapid means of surface colonisation. Bacteria twitch by extending their type IV pili, attaching to the surface, and then retracting, bringing the whole cell closer to the point of contact. This motion is powered by the ATPase PilT.

In Chapter 2 the flagellum and type IV pilus biosynthetic gene clusters are compared between strains of *P. ananatis* and closely related enterobacterial strains. The four fully annotated and sequenced *P. ananatis* strains used in this study were isolated from various different sources and provided a greater understanding of how *P. ananatis* exploits its flagella and type IV pili to infect such a wide variety of hosts. While Chapter 3 focuses on the creation of four motility mutants and their respective complements in *P. ananatis* LMG20103, Chapter 4 consists of an array of tests and assays comparing the mutants to the wild-type strain to elucidate the role of swimming and twitching motility in the colonisation and infection of *P. ananatis* in onions. Chapter 5 is a published article titled, "Draft genome sequences of the onion centre rot pathogen *Pantoea ananatis* PA4 and maize brown stalk rot pathogen *P. ananatis* BD442." Both strains are South African isolates and were sequenced using the Illumina HiSeq 2500 platform.

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A greater understanding of how *P. ananatis* uses motility to target tissues and infect its host plant increases the currently limited body of knowledge available to develop strategies to limit the damage caused by this pathogen to agronomic crops in plantations and nurseries.

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CHAPTER 1

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CHAPTER ONE

MOTILITY AS A MEANS OF COLONISATION AND AS A VIRULENCE FACTOR IN PLANT PATHOGENIC BACTERIA

1.1 INTRODUCTION

The accumulative surface of plant leaves (approximately 1.2×10^9 km^2) forms the largest biosphere-atmosphere interface known to man (Beattie, 2004). This phyllosphere plays host to numerous microorganisms, including bacteria, filamentous fungi and yeasts. The bacteria that are found in this habitat are themselves very diverse, ranging from those that have a benign or beneficial effect on the host plant, to those that are aggressive pathogens that either wound or kill the host plant. The phyllosphere bacterial population consist of both epi- and endophytes (Morris *et al*., 2004).

Epiphytes are able to carry out their entire life cycle on the surface of plant leaves and have consequently adapted to those environmental conditions found in the phytosphere. The epiphytic environment is both varied and taxing. The population size of bacteria is largely dependent on environmental conditions and stresses, as well as the ability of the leaf to shield the bacteria from such stresses (Lindow and Leveau, 2002). Epiphytic bacteria are often found in protected sites on the surface of leaves such as along veins or between epidermal cells. A large number of bacterial cells can also be found near plant organs that provide direct access to the interior of the leaf such as trichomes, stomata and hydathodes (Manceau and Kasempour, 2004). Stomata are usually closed against microbial invasion, but bacteria such as *Pseudomonas syringae* produce a compound known as coronatine that promotes the re-opening of the stomata, allowing the bacteria entry to the interior of the leaf (Schulze-Lefert and Robatzek, 2006). They can also invade leaf tissue through natural openings or wounds in the cuticle. The majority of epiphytic bacteria are found on the underside of the leaf, as opposed to the upper surface, as this surface is more protected from the environment and the majority of stomata are located there (Leben, 1988; Surico, 1993; Donderski and Kalwasinska, 2002; Monier and Lindow, 2004). Endophytic bacteria usually colonise the inside of leaves, such as in the intercellular spaces (Manceau and Kasempour, 2004). Endophytes are relatively well protected from environmental stresses, but they have to be able

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to overcome the host plant's defence system (Morris *et al*., 2004). Some bacteria such as *Pantoea ananatis* and *P. syringae* can have both epi- and endophytic lifestyles (Sabaratnam and Beattie, 2003).

Epiphytic populations are most often dominated by Gram-negative bacteria. Epiphytic Gram-positive bacteria are usually found within biofilms and cell aggregates, and only a small percentage of the single cell population on the leaf surface are Gram-positive (Morris and Monier, 2003). This is most likely due to the fact that Gram-positive bacteria are even more susceptible to the harsh environment on a leaf surface than Gram-negative bacteria, as they lack the outer membrane present in the latter (Costerton *et al.*, 1974). The genera that usually make up the largest percentage of epiphytic communities are *Pseudomonas*, *Xanthomonas*, *Erwinia* and *Pantoea* (Lindow and Brandl, 2003). It has also been observed that pathogenic strains of leaf-associated bacteria are generally more prevalent than the non-pathogenic strains, but only under dry conditions (Wilson *et al*., 1999).

The phyllosphere is still a largely unexplored field when compared to other bacterial habitats. This review focuses on revealing more of the epiphytic nature of bacteria, particularly those that have the potential to cause plant diseases. It takes a closer look at *P. ananatis*, which is naturally epiphytic on numerous plant species and the focus of this thesis, before moving on to bacterial motility in general.

1.2 LIFE IN THE PHYLLOSPHERE

The leaf surface is known to be a very hostile environment and is generally unfavourable to bacterial growth. It is often directly exposed to ultra violet (UV) irradiation, fluctuating temperatures, low nutrient availability and a fluctuating, and often very low, water availability that results in dehydration, high osmolarities, and high concentrations of toxins, antimicrobials and other inhibitory compounds (Lindow and Brandl, 2003). Bacteria that are found growing on leaf surfaces, therefore need to have high levels of UV tolerance and efficient DNA repair mechanisms (Jacobs and Sundin, 2001), as well as having to be able to requisition nutrients from their barren environment (Beattie and Lindow, 1995). It has been said that leaf surfaces are the "breeding grounds" of genetic exchange, and also of microbial diversity (Lindow and Leveau, 2002) as epiphytes display high rates of plasmid transfer

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within the community, exchanging and sharing those genes that code for traits that allow them to survive on the hostile leaf surface. The genes within a community become extensively mixed after an extended period of time, which makes both the genotype and the phenotype of epiphytic bacteria relatively unstable (Romantschuk *et al*., 2004).

The ability of bacteria to cope with environmental stresses in the phyllosphere is a measure of their epiphytic fitness. A wide variety of traits add to the bacteria's epiphytic fitness in small increments. Some traits such as siderophore (for iron sequestration) and antibiotic production, as well as ice nucleation activity, do not substantially contribute to the cell's overall fitness. On the other hand, traits such as motility, adhesion, UV-damage mutagenic repair and the production of exopolysaccharides (EPS), have a considerable effect on the cell's fitness (Marco *et al*., 2005). Epiphytes are able to protect themselves from their environment in numerous ways. Many epiphytes are pigmented; this is most likely to protect the bacteria from UV radiation (Jacobs and Sundin, 2001). Also, there seems to be a marked difference in both community size and diversity, depending on the season. There is greater community diversity during the cooler, wetter months (Thompson *et al*., 1993). Many bacteria are able to subtly alter their environment to increase their chances of survival, for example, the production of biosurfactants increase the solubility and diffusion properties of many nutrients and gasses, allowing bacteria to have greater access to them (Lindow and Brandl, 2003). Biosurfactants can also act as emulsifiers, thus releasing certain constituents of the cuticle to be used as carbon sources by the bacteria (Ron and Rosenberg, 2001). Certain compounds such as syringomycin and syringopeptin produced by *P. syringae*, are toxins that increase the bacteria's virulence, as well as ultimately inducing plant cell lysis, releasing the nutrients that are trapped within the plant cells (Hutchinson *et al*., 1995). Another compound produced by many epiphytic bacteria, indole-3-acetic acid (IAA), is a plant growth regulator. The production of IAA in low concentrations results in plant cells releasing saccharides, thereby increasing nutrient availability to the bacterial cells on the leaf surface (Lindow and Brandl, 2003). The production of IAA in *Pantoea agglomerans* is induced under conditions of low water availability (Brandl and Lindow, 1997), most likely providing a mechanism to supply bacteria with the necessary nutrients in times of drought or starvation. The production of EPS and rhamnolipids increases the ability of bacteria to swarm across a surface (Fraser and Hughes., 1999; Caiazza *et al*., 2005; Herrera *et al*., 2008). Antibiotics are produced to deter competing bacterial and fungal species, thus allowing the bacteria to occupy their niche with less competition (Haas and Keel, 2003).

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Another survival mechanism of epiphytes is to grow together in large aggregates or biofilms as opposed to single cells; usually in protected and favourable areas on the leaf such as along veins or near trichomes and stomata (Danhorn and Fuqua, 2007). The formation of aggregates and biofilms not only offers bacteria protection from a number of environmental stresses (Lindow and Brandl, 2003), but it also encourages the use of quorum sensing, which plays a vital role in regulating numerous epiphytic fitness and virulence genes in bacteria (Morris *et al*., 2004). Within biofilms bacteria are able to efficiently modify their environment so as to make it more suitable for survival and proliferation. The EPS, which makes up the matrix of the biofilm, is able to protect the cells within the biofilm from desiccation and reactive forms of oxygen (Johnson *et al*., 2000). It is also able to concentrate the nutrients from the surrounding environment, increasing the general epiphytic fitness of the bacterial community that makes up the biofilm (Lindow and Brandl, 2003). EPS can even be found surrounding single cells, protecting them from desiccation and harmful UV rays (Beattie and Lindow, 1995). Auxins and biosurfactants are usually produced within the confines of biofilms, as the biofilm concentrates these compounds, thereby increasing their efficacy (Morris *et al*., 2004).

The leaf itself provides some protection for the bacteria. It is surrounded by a very thin laminar layer that traps moisture released though the stomata. This moisture may provide bacteria with the necessary hydration during periods of drought (Lindow and Brandl, 2003). It is thought that bacteria are often found growing at the base of trichomes due to the nutrients that leak through the cracks in the cuticle layer at this location (Beattie and Lindow, 1995). Bacteria are also found in depressions in the cuticle, or beneath the cuticle, where they are offered some protection against the elements (Beattie, 2004). The leaf cuticle plays an important role in the survival of epiphytic populations as it affects the initial distribution of bacterial cells across the leaf surface by influencing insect, water and aerial distribution (Beattie, 2004). The leaf cuticle not only acts as a physical barrier to prevent the bacteria from penetrating the leaf, but also plays a role in dictating how much water is available on the leaf surface. This, in turn, affects the concentration of nutrients on the leaf surface, as well as the motility of bacteria. The physical barrier that the cuticle presents, prevents the diffusion of nutrients from the plant leaves to the upper surface, thus limiting the amount of nutrients available to the bacterial cell. The presence of a cuticle also affects quorum sensing, as many of the signalling molecules are absorbed by the waxes of the cuticle (Cha *et al*., 1998). However, cuticles do not always deter bacteria. Certain bacteria such as *Bacillus subtilis* have

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been found living embedded in the actual cuticle of the plant where they are protected from the harsh environmental conditions on the leaf surface (Beattie, 2004). Other organisms such as *P. syringae* produce cutinases and are therefore able to penetrate the cuticle (Fett *et al*., 2005). The production of cutinases, however, is a relatively rare trait in bacteria (Beattie, 2004).

Bacteria that live and thrive in the phyllosphere have adapted in such a way as to be able to overcome the many hurdles that prevent other bacteria from making the phyllosphere their home. *P. ananatis* is an example of a successful epiphyte, although it is also found in a wide variety of other environments (Rauch *et al*., 2006; Hara *et al*., 2012).

1.3 *PANTOEA ANANATIS*

Pantoea ananatis is a phytopathogen on a broad range of plant hosts, including *Eucalyptus* (Coutinho *et al*., 2002), onion (Gitaitis *et al*., 2002; Walcott *et al*., 2002), maize (Paccola-Meirelles *et al*., 2001; Goszczynska *et al*., 2007), pineapple (Serrano, 1928), rice (Watanabe *et al*., 1996), cantaloupe fruit (Bruton *et al*., 1991), sudan grass (Azad *et al*., 2000), watermelon (Walcott *et al*., 2003) and honeydew melon (Wells *et al*., 1987). *P. ananatis* is a necrogenic plant pathogen that causes plant cell death, although it is not always pathogenic towards its host plant. This bacterium is found as both an epiphyte and endophyte on a wide variety of plants that do not display symptoms, such as crabgrass, yellow nutsedge, cowpea, Bermuda grass and soybean (Gitaitis *et al*., 2002). Although it is usually isolated from soil and plant sources, *P. ananatis* is capable of proliferating in a wide variety of environments that include aviation fuel tanks (Rauch *et al*., 2006), sorghum fermentations (Mohammed *et al*., 1991; Watanabe and Sato, 1999; Wells *et al*., 2002) and it has been found associated with insect hosts including ticks, fleas, mulberry pyralids and tobacco thrips (Murrell *et al*., 2003). It has been identified as the causative agent of bacteraemia in immune-compromised patients (De Baere *et al*., 2004; Van Rostenberghe *et al*., 2006). *P. ananatis* has also been used as a biological control agent due to its ability to produce ice nucleation proteins. This means that it can be used against common insect pests, such as mulberry pyralids, as the bacterium infects the gut of the insect larvae, causing the insect's resistance to cold to be lowered (Watanabe and Sato, 1999). However, *P. ananatis* can also inflict frost injury on its host plant as has been observed in mulberry and tea plants (Takahashi *et al*., 1995). This specific trait has been

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exploited in the food industry where they use *P. ananatis* to freeze-dry food products (Watanabe and Arai, 1994), and to freeze certain foods while still maintaining a desirable texture (Zasypkin and Lee, 1999). As an endophyte, *P. ananatis* also displays beneficial characteristics by fixing nitrogen in dune grasses (Dalton *et al*., 2004), promoting plant growth through the production of compounds such as IAA and inducing resistance towards other pathogens such as *Erwinia amylovora* and *Xanthomonas axonopodis* pv. *vesicatoria*, as *P. ananatis* has both antibacterial and antifungal properties (Kang *et al*., 2007; Walterson *et al*., 2014).

At present, it is still unclear how *P. ananatis* is transmitted from host to host. It has been proposed that it enters the host through its flowers, insect-associated wounds, mechanical-injury wounds (Serrano, 1928) and plant-to-plant contact during strong winds (Azad *et al*., 2000). It has also been shown to be transmitted by insects such as tobacco thrips (Wells *et al*., 2002; Gitaitis *et al*., 2003), mulberry pyralids (Takahashi *et al*., 1995) and brown planthoppers (Watanabe *et al*., 1996). This bacterium can also be seedborne (Ferreira *et al*., 2008; Walcott *et al*., 2002; Coutinho and Venter, 2009).

In South Africa specifically, *P. ananatis* infects *Eucalyptus* seedlings, maize and onion plants (Coutinho *et al*., 2002; Goszczynska *et al*., 2006a; Goszczynska *et al*., 2007). *P. ananatis* was first shown to cause disease in *Eucalyptus* trees in 1998, when it was repeatedly isolated from the infected tissue of trees suffering from leaf blight and dieback of young shoots. This disease resulted in the stunted growth of infected trees. The symptoms in *Eucalyptus* seedlings usually include leaf spots and dieback at the tip of the leaf. Initially the leaf spots are water-soaked and it is not uncommon for them to join together to form larger spots or lesions (Coutinho *et al*., 2002). The lesions are usually concentrated along the main veins of the leaf and the leaf petioles often become necrotic and, as a result, the infection is hypothesised to spread from the petiole to the main veins where it then spreads to the surrounding tissue. The necrotic petioles result in the leaves dying and falling off, and repeated infection leads to the trees having a scorched appearance and stunted growth. *P. ananatis* outbreaks in plantations usually result in either the death of the young trees, or they grow to have multiple stems (Coutinho *et al*., 2002). The severity of the disease increases in conditions of high humidity and moderate temperatures (Coutinho *et al*., 2002; Schwartz *et al*., 2003). The disease can infect a variety of *Eucalyptus* species, clones and hybrids, and can therefore cause substantial economic losses in plantations and nurseries. This is especially

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disconcerting as commercially grown *Eucalyptus* trees account for more than 50% of all newly afforested areas in South Africa and is the most widely planted hardwood crop in the world (Coutinho *et al*., 2002). *Eucalyptus* species have been planted on 20 million ha worldwide and are used to make paper, pulp or timber products (Mizrachi *et al*., 2010). Currently, the only method of controlling *P. ananatis* in *Eucalyptus* plantations and nurseries is through the use of resistant clones (Coutinho and Venter, 2009).

P. ananatis was first isolated from maize in Brazil in 1982 where it led to a reduced plant cycle, leaf senescence, and a decrease in grain size and weight (Paccola-Meirelles *et al*., 2001). Yield losses in maize crops due to *P. ananatis* infection are sometimes as high as 63% (Lana *et al*., 2012). Symptoms include chlorosis and water-soaked spots that later become necrotic lesions, stunted growth and a vertical crack at the first internode (Paccola-Meirelles *et al*., 2001; Perez-y-Terron *et al*., 2009; Lana *et al*., 2012). *P. ananatis* was also found to cause brown stalk rot of maize in South Africa in 2004 (Gosczcynska *et al*., 2007). Maize is the most important grain crop in South Africa as it forms part of the staple diet of the nation, as well as being the major grain crop used in animal feeds (National Department of Agriculture, 2003). On average, 2.7 million ha of maize are cultivated in South Africa each year (National Department of Agriculture, 2003; CEC Maize, 2014). As with *Eucalyptus*, the only current method of controlling white spot disease in maize is through the use of resistant strains and cultivars (Lana *et al*., 2012).

The disease caused by *P. ananatis* on onions is commonly known as centre rot and was first observed on onion in Georgia in 1997 (Gitaitis and Gay, 1997). Symptoms include water-soaked lesions on the leaves, wilting and bleaching of the leaves, and rotting of the bulbs. Diseased onions cannot be harvested as easily as healthy onions as the leaves of the onion tear away, leaving the bulb in the ground (Gitaitis *et al*., 2002). It has been shown that *P. ananatis* is both seed-borne and seed-transmitted in onions. *P. ananatis* was first isolated from diseased onions in South Africa in 2006 (Gosczcynska *et al*., 2006a).

The pathogenicity of *P. ananatis* is poorly understood, however, due to the increasing availability of sequenced *P. ananatis* genomes, the mechanisms by which *P. ananatis* is able to infect and induce disease on various hosts is slowly being revealed. Comparisons to well-characterised plant pathogens such as *E. amylovora*, *Pectobacterium carotovorum* and *Dickeya dadantii* have shown that orthologues of pathogenicity determinants, such as flagella

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and fimbriae biosynthetic proteins, non-fimbrial adhesins, ice nucleation proteins and potential cell wall degrading enzymes are encoded on the genome of *P. ananatis* (de Maayer *et al*., 2014). It is thought that the pathogenesis of *P. ananatis* is largely attributed to the synthesis of EPS and the possible production of toxins which is regulated through quorum sensing (Morohoshi *et al*., 2007). *P. ananatis* also has a type VI secretion system which may play a role in pathogenicity on both plant and animal hosts (de Maayer *et al*., 2011; Shyntum *et al*., 2014).

1.3.1 Identification and detection of *Pantoea* species

P. ananatis belongs to the class Gamma-proteobacteria and the family *Enterobacteriaceae*. It is Gram-negative, motile, rod-shaped, facultatively anaerobic and is usually between 1 to 2 µm in size. It was originally classified in the genus *Erwinia*, as *E. ananas*, which is synonymous to *E. uredovora* (Mergaert *et al*., 1993). The type strain of *P. ananatis*, LMG2665^{T}, was first isolated from pineapple in 1928 (Serrano, 1928). *P. ananatis* produces a yellow pigment when grown on nutrient agar (Wells *et al*., 1987). The production of pigments in the genus *Pantoea* is a protective mechanism as the colour protects the cells from harmful UV radiation, as well as being an important part of the antioxidant pathway (Mohammadi *et al*., 2012). Some other distinguishing characteristics of *P. ananatis* include the production of acid from melibiose, inositol, cellobiose and glycerol (Bruton *et al*., 1991). A selective medium, PA20, has been developed for the species, which greatly increases growth efficiencies of *P. ananatis*, while simultaneously suppressing the growth of almost all saprophytic organisms (Goszczynska *et al*., 2006b).

P. ananatis can be identified using numerous phenotypic tests, including API 20E and BIOLOG, as well as genotypic tests including 16S rDNA or tRNA sequencing (Brady *et al*., 2007; De Baere *et al*., 2004). However, these tests can only adequately identify members of this species to the genus level. 16S rDNA sequencing is often complemented by PCR amplification of the ice nucleation gene *inaA*, which is species-specific (Lana *et al*., 2012). Fluorescent amplified fragment length polymorphism (FAFLP) and multi-locus sequence analysis (MLSA) can also be used for identification of *P. ananatis* to the species level (Brady *et al*., 2007; Brady *et al*., 2008). Recently the use of matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) has been shown to rapidly and accurately distinguish between species of *Pantoea* (Rezzonico *et al*., 2010). An

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immunomagnetic separation-PCR (IMS-PCR) has been successfully used to detect *P. ananatis* in plant tissue, specifically in onion seeds. This method is advantageous over other conventional methods, as plant tissue contains a substantial amount of compounds that inhibit DNA amplification. IMS-PCR makes use of small polystyrene beads that are superparamagnetic and have been coated with antibodies that are specific to the bacterium that is being tested for. The beads thus select for and concentrate target cells from which DNA is released and which can be used as template DNA for amplification during the PCR reaction (Walcott *et al*., 2002).

In a study conducted by Kido *et al*. (2010), ninety-six strains of *P. ananatis* where divided into three distinct groups based on a welsh onion stabbing assay, tobacco infiltration test, as well as the presence or absence of the IAA genes *iaaM, iaaH* and the cytokinin biosynthesis gene, *etz*. Group 1 consisted of all the strains that caused lesions on onions and induced a hypersensitive response in tobacco although they lacked the *iaaM, iaaH* and *etz* genes. Group 2 included all strains that were isolated from melon and had all three specified genes but were unable to form lesions or reactions on onion and tobacco. Strains that lacked both the three genes and were unable to form lesions were placed in group 3. These tests and assays may serve as a rapid assay to predict the behaviour of *P. ananatis* strains on important crops such a rice. This is demonstrated by further results obtained by Kido *et al*. (2010), as they observed that group 1 caused palea browning on rice but did not cause internal fruit rot on melon. Group 2 were capable of the reverse as they caused internal fruit rot on melons but showed no disease symptoms on rice. Group 3 were non-pathogenic. The pathogenic variation of *P. ananatis* can therefore be predicted by a hypersensitive response assay on tobacco and a PCR detection of any one of the *iaaM, iaaH* and *etz* genes (Kido *et al*., 2010).

1.4 BACTERIAL MOTILITY

1.4.1 Swimming motility

Motility is advantageous to bacteria as it enables them to gain better access to nutrients, allows them to avoid toxic substances or deterrents, move to more sheltered and hospitable environments, find and colonise preferred hosts and tissues and also enables them to disperse more effectively (Ottemann and Miller, 1997; Demir *et al*., 2011; Ichinose *et al*., 2013).

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Although other forms of motility are available to bacteria, the most common forms are those that require extracellular organelles, namely flagella and pili. Swimming, twitching and swarming motility are most commonly used by bacteria.

Swimming motility occurs through the use of rotating flagella (Shen and Ronald, 2002). Flagella are central to survival and proliferation of bacteria, and thus more than 80% of all known bacterial species have flagella, and even several species of Archaea are flagellated (Thomas *et al*., 2001). Within bacterial species there can be variation in the number, structure and location of the flagella with some species being mono- or lophotrichous, while others are peritrichously or amphitrichously flagellated (Figure 1.1) (Soutourina and Bertin, 2003; Kirov, 2003). Flagellum-mediated motility is faster than other forms of bacterial motility, including twitching motility. Using flagella, *Escherichia coli* can swim up to 25 µm/sec, whereas twitching motility in *Myxococcus xanthus* and *Pseudomonas aeruginosa* results in a slower movement of about 0.4 µm/sec and 0.6 µm/sec, respectively (Alm and Mattick, 1997; Sun *et al*., 2000).

In many bacteria such as *P. aeruginosa*, *Pseudomonas putida* and *Ralstonia solanacearum*, flagella play an important role in attachment and biofilm formation (O'Toole and Kolter, 1998; Turnbull *et al*., 2001; Kang *et al*., 2002; Meng *et al*., 2011). Flagellar motility is important in bringing the bacterial cell close enough to the surface to overcome the repulsive forces that naturally exist between the cell and the surface, allowing the bacteria to attach (O'Toole and Kolter, 1998). It is likely that flagella play a role during the initial spread of the biofilm (Pratt and Kolter, 1998; Ramos *et al*., 2004). In plant pathogens, motility is important in the early phases of infection, predominantly through the ability to respond to chemotactic signals and adherence. After the initiation of infection, motility is often down-regulated (Josenhans and Suerbaum, 2002; Soutourina and Bertin, 2003; Meng *et al*., 2011). Flagella have also been implicated in playing a role in invasion during infection (Kirov, 2003).

The flagella are coupled to a chemotaxis protein cascade that allows bacteria to move in response to environmental signals (Ramos *et al*., 2004). When flagella turn in an anticlockwise direction, the cells are propelled forward (Macnab, 2003). This is commonly known as a "run" and lasts for a few seconds (Ramos *et al*., 2004). Cells with multiple flagella co-ordinate their flagella in such a way that they form a bundle and work together. Runs are more frequent as the bacterium approaches attractants such as amino acids or sugars

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(Kirov, 2003). When the bacterium receives a signal from the chemotaxis cascade to change direction the flagella rotate in a clockwise direction, the flagella bundle flies apart and the cell tumbles. This usually lasts for a fraction of a second (Ramos *et al*., 2004). Tumbles increase as bacteria near repellants (Kirov, 2003). Anticlockwise rotation is then resumed and the cells will once more move forward in a new direction (Macnab, 2003; Ramos *et al*., 2004). Cells with a single flagellum move forward during a run but instead of tumbling, the flagellum stops rotating and the cells are re-orientated by Brownian motion. When the flagellum resumes rotation the cell moves forward in a new direction (Madigan *et al*., 2003).

1.4.1.1 Chemotaxis

Chemotaxis is the ability of bacteria to sense and subsequently move either up or down a chemical gradient; towards attractants and away from repellents. Chemotaxis is important for both motility and pathogenesis, and allows bacteria to adapt to changing environmental conditions (Soutourina and Bertin, 2003). In the phytosphere, chemotaxis allows bacterial cells to locate, invade and colonise plant tissues effectively (Yao and Allen, 2006). Bacteria are also, by means of chemotaxis, able to sense wounds and injuries in their host plants and swim toward these openings to gain entry into the plant (Szurmant and Ordal, 2004). Chemotactic bacteria are attracted to simple sugars, amino acids, organic acids, aromatic compounds and secondary metabolites that are exuded from injuries on the plants and glandular trichomes whereas they are repelled by compounds that are not beneficial to their growth such as toxins, antimicrobials and reactive oxygen species (Brencic and Winans, 2005).

R. solanacearum is specifically attracted to amino acids, organic acids and exudates from its host plant, tomato. *R. solanacearum* was observed to be attracted to tomato exudates three times more strongly than to rice plant exudates (Yao and Allen, 2006). Bacteria such as *Xanthomonas oryzae* pv. *oryzae* swim toward exudates from susceptible rice plants and avoid resistant plants (Shen and Ronald, 2002). However, the response of bacteria to chemoattractants may vary between species, and even between strains of the same species (Yao and Allen, 2006).

Bacteria can sense external stimuli either directly through binding to the stimulus, or indirectly through responding to a metabolic change brought on by the stimulus (Mazzag *et*

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al., 2003). The stimuli bind to methyl-accepting chemotaxis proteins (MCPs), which are receptors in the cell membrane. Four different MCPs that react to specific stimuli have been identified in *E. coli*, namely the Tar (aspartate), Trg (ribose and galactose), Tsr (serine) and Tap (peptides) receptors (Grebe and Stock 1998; Taylor *et al*., 1999). *Salmonella enterica* lacks the Tap receptor and has a Tcp receptor instead, which mediates taxis to citrate (Iwama *et al*., 2006). The binding of the stimuli changes the conformation of the MCPs and this excites the CheA protein (Taylor *et al*., 1999; Kirov, 2003; Macnab, 2003; Madigan *et al*., 2003). CheA is a histidine kinase that is autophosphorylated and is coupled to the MCPs by CheW (Yao and Allen, 2006). CheW autoregulates CheA (Macnab, 2003; Subramanian and Nott, 2011). The phosphorylated CheA then transfers its phosphate group to CheY which is a cytoplasmic response regulator (Madigan *et al*., 2003; Yao and Allen, 2006). Once phosphorylated, CheY is able to bind to the flagellar switch and is responsible for causing the flagellum to rotate in a clockwise motion (Taylor *et al*., 1999; Macnab, 2003; Madigan *et al*., 2003; Alexandre *et al*., 2004; Yao and Allen, 2006; Subramanian and Nott, 2011). The phosphorylated CheY protein is gradually dephosphorylated by the phosphatase CheZ, resulting in CheY no longer being able to bind to the flagellum, causing it to return to a anticlockwise rotation (Macnab, 2003; Subramanian and Nott, 2011). As stated previously, flagella rotating in a anticlockwise direction results in a bacterial cell moving forward, whereas a clockwise rotation results in a change in the swimming direction (Mazzag *et al*., 2003). Attractants decrease the rate of autophosphorylation of CheA, whereas repellants increase it (Madigan *et al*., 2003).

The cell is able to adapt to changes in the concentration of the attractant or repellent as the chemoreceptors are methylated or demethylated by the CheR methyltransferase and CheB methylesterase, respectively (Taylor *et al*., 1999). Phosphorylated CheA is also able to phosphorylate CheB, which is similar to CheY in that it is also a response regulator. When phosphorylated, the CheB protein is activated. The MCPs are continually methylated by CheR, which adds a methyl group to each MCP using *S*-adenosylmethionine as a methyl donor. This means that the MCPs can be "reset" even if the concentration of the attractant or repellent remains unchanged (Madigan *et al.*, 2003). Consequently, if the concentration of an attractant is consistently high, the phosphorylation levels of CheA, CheY and CheB will remain low. This results in the cell continuing to swim and the increased methylation of the MCPs. Once fully methylated, the MCPs are no longer able to respond to the attractant, and the phosphorylation of CheA and CheB increases, causing the cell to tumble and the MCPs to

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be demethylated. When the chemotactic stimulus is a repellent the opposite is true as fully methylated MCPs respond best to repellants (Madigan *et al*., 2003; Alexandre *et al*., 2004).

The important role of chemotaxis in plant colonization and pathogenesis has been demonstrated in a range of plant-associated bacteria. *R. solanacearum cheA*$^{-}$ and *chew*$^{-}$ mutants were completely non-chemotactic, demonstrating that chemotaxis is dependent on these two proteins. The mutants also showed reduced virulence (Yao and Allen, 2006). Chemotaxis is necessary for *Pseudomonas fluorescens* to effectively colonise tomato roots (de Weert *et al*., 2002) and *Agrobacterium tumefasciens* mutants deficient in chemotaxis were non-pathogenic on pea plants (Hawes and Smith, 1989).

1.4.1.2 Flagellum structure

The flagellum essentially consists of a motor, basal body, hook and filament (Figure 1.2). The motor is divided into the stator and the rotor. The stator, consisting of MotA and MotB proteins, is stationary and found in the peptidoglycan layer, whereas the rotor consists predominantly of FliG protein. FliG, along with FliM and FliN, interacts with the chemotaxis proteins and can switch the flagellum from a clockwise to an anticlockwise rotation (Francis *et al*., 1994). Along with the stator, the rotor generates torque which drives the flagellum (Macnab, 2003; Ito *et al*., 2004). MotA is involved in transporting protons across the membrane and using the protons to generate torque, thus establishing the proton motive force that allows the flagellum to rotate. MotB anchors the flagellar motor in the membrane and, along with MotA, forms the proton channel that allows protons to cross the membrane (DeRosier, 1998; Madigan *et al*., 2003). Not all flagellated bacteria have a MotAB motor. MotPS is orthologous to MotAB and mutants are non-motile. *Bacillus pseudofirmus* and *Bacillus halodurans*, which have a MotPS motor, are alkaliphilic and their flagellar motor is dependent on a sodium motive force as opposed to the more common proton motive force. *B. subtilis* has both a MotAB and a MotPS system, although it still has a fully functioning flagella in MotPS mutants (Ito *et al*., 2004).

The motor is linked to the basal body which consists of a membrane-supramembrane (MS) ring that is located in the membrane, a peptidoglycan (P) ring found in the periplasmic space and a lipopolysaccharide (L) ring that is situated outside the cell (Kirov, 2003). A rod travels through the centre of all three rings. The basal body transmits the torque it receives from the

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motor to the hook and subsequently the filament. It also anchors the flagellum in the cell membrane and forms a channel for the export of flagellar constituents (Kirov, 2003). The hook not only transmits the torque from the motor to the filament, but also co-ordinates bundle formation in bacteria that have multiple flagella (Fraser *et al.*, 1999; Macnab, 2003). The hook consists predominantly of FlgE protein (Macnab, 2003). Hook-associated proteins (HAPs) include FlgK and FlgL (Fraser *et al*., 1999). Without HAPs the flagella are unable to fully assemble (Homma *et al*., 1990; Ikeda *et al*., 1993; O'Toole and Kolter, 1998).

The filament is made up of subunits of flagellin proteins, known in *Enterobacteriaceae* as FliC (Kirov, 2003), which are conserved among a wide range of bacterial species (Ramos *et al*., 2004). The filament is long, thin and helical in shape, and can be up to 15 µm long (Macnab 2003; Ramos *et al*., 2004). There is a 3-nm channel extending down the centre of the filament from the MS ring to the tip of the filament. This channel plays an important role in the type III flagellar export pathway. Flagellin is added to a growing flagellum by being secreted down the centre of the filament and assembled at the distal end, but the flagellum also exports non-flagellar proteins such as virulence-associated phospholipases (Kirov, 2003). The filament tip is capped by a capping protein, FliD (Fraser *et al*., 1999; Macnab, 2003). The capping protein is responsible for polymerisation of the flagellin subunits and for preventing the flagellin from escaping into the extracellular environment (Ramos *et al*., 2004). The assembly of the basal body and hook requires a capping protein as well (Kirov, 2003).

1.4.1.3 Genomic organisation and regulation

In *E. coli*, which has peritrichous flagella, there are approximately fifty genes coding for flagellar synthesis and functionality, and they are organised into fifteen operons that are clustered in several regions throughout the genome. Cluster I includes some of the genes that encode the flagella structural genes, whereas cluster II includes genes encoding proteins for the regulation of flagellar assembly, the chemotactic genes and the flagella motor genes *motA* and *motB*. Cluster III contains the flagellar structural genes, export apparatus proteins and the flagellar-specific sigma factor; σ^{28} (Soutourina and Bertin, 2003).

The synthesis and functioning of bacterial flagella places a large metabolic burden on the cell as it uses about 2% of all biosynthetic expenditure. Flagella may also induce an immune response in the host organism. The expression of flagellar genes is thus highly regulated

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through a gene hierarchy where the expression of one gene regulates the transcription of the next level of genes (Lehti *et al*., 2012). At the top of the hierarchy is a master operon, which controls the transcription of all other flagella genes. The master regulator in *Enterobacteriaceae* consists of the genes *flhDC*, while *fleQ* and *flrA* fulfil this role in *P. aeruginosa* and *Vibrio cholerae*, respectively (Soutourina and Bertin, 2003). The FlhDC complex is negatively regulated by MotN. *motN* mutants in *R. solanacearum* are hypermotile and have reduced virulence (Meng *et al*., 2011). Class II consists of genes encoding the basal body and export system, as well as $\sigma^{28}$and FlgM which positively and negatively regulate the genes of class III, respectively. Class III codes for the flagellar filament and proteins that are associated with the hook, motor and chemotaxis (Soutourina and Bertin, 2003). In *E. coli*, Mat pili are able to repress the expression of flagella. Mat pili are involved in adhesion and biofilm formation so their ability to repress flagella demonstrates how cells are able to "switch off" their motility when they firmly attach to a surface (Lehti *et al*., 2012).

Environmental factors also play a role in the regulation and synthesis of flagella. Flagella biosynthesis in *E. coli* is inhibited by catabolite repression in the presence of D-glucose, by high temperatures or high salt concentrations, extreme pH and when in the presence of DNA gyrase inhibitors or low molecular mass alcohols (Li *et al*., 1993). Low oxygen concentrations have been shown to induce flagellar synthesis (Landini and Zehnder, 2002). Flagella in *S. enterica* are affected by pH and temperature, whereas *Campylobacter coli* flagella are regulated by temperature, pH and the concentration of inorganic salts and divalent ions (Soutourina and Bertin, 2003). *X. oryzae* pv. *oryzae* has a single flagellum that is induced under limited nutrient conditions (Shen and Ronald, 2002). Swimming motility is also influenced by water availability, nutrient availability, polysaccharide and surfactant production, and temperature (Harshey *et al*., 2003).

1.4.1.4 The role of flagella in pathogenesis

Several phytopathogenic studies have unequivocally demonstrated that flagella are required for swimming motility and that the lack of motility results in reduced virulence. Aflagellate *A. tumefaciens* and *P. syringae* pv. *phaseolicola* mutants exhibited reduced virulence on sunflowers and bean leaves, respectively (Panopoulos and Schroth, 1974; Shen and Ronald, 2002). A similar result was observed when flagellin (*fliC*) and hook-associated protein *flgE* genes in *X. axonopodis* pv. *citri* were deleted through knock-out mutagenesis. Strains

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displayed reduced virulence on citrus leaves and their swimming abilities were reduced. FliC was found to be important for normal flagellum function, virulence and growth in *Edwardsiella tarda* (He *et al*., 2012). It was also shown that the flagellum of *X. axonopodis* pv. *citri* plays a role in biofilm development (Malamud *et al*., 2011). Knock-out mutagenesis of *flgK*, which codes for a hook-associated protein, resulted in incomplete assembly of the flagella of *E. coli*, *Salmonella typhimurium* and *P. aeruginosa*, and the mutants were thus non-motile (Homma *et al*., 1990; O'Toole and Kolter, 1998). Mutations in the *motA* and *motB* genes in *E. coli* resulted in complete flagella synthesis, but the flagella were non-functional, i.e. the cells were paralysed. *cheA-Z*$^{-}$ mutants were shown to be motile but not chemotactic, although the bacteria were still capable of biofilm formation. It could thereby be inferred that motility, and not chemotaxis, is important in biofilm formation (Pratt and Kolter, 1998; Ramos *et al*., 2004).

1.4.1.5 Flagellum anomalies

Some bacterial species, such as *S. enterica* (Ikeda *et al*., 2001; Bonifield and Hughes, 2003), *Campylobacter jejuni* and *C. coli* (Harris *et al*., 1987; Park *et al*., 2000) contain multiple copies of the *fliC* gene, coding for distinct flagellin (FliC) proteins. These can partake in the phenomenon of phase variation, which can be defined as a "variation in flagellar antigen characterized by the appearance of two or more alternative types of flagellar antigen" (Iino, 1969). The flagellin proteins are highly immunogenic and can be recognized by the host defence system (Yang *et al*., 2012). In response, the pathogen can affect the transcription of an alternative flagellin gene (Bonifield and Hughes, 2003).

S. enterica serovar Typhimurium is able to alternatively express two antigenically distinct flagellin subunits, namely FliC and FljB (Bonifield and Hughes, 2003) which affects the bacterium's pathogenicity in a mammalian host, for example, the FliC^{+} phase confers a selective advantage to bacteria in the murine typhoid infection model, however, this is not the case for the induction of enteropathogenesis (Ikeda *et al*., 2001). The N- and C-terminals of the flagellin proteins are identical, however, the centre-region, which correlates with the surface-exposed amino acids of the folded protein, are different (Bonifield and Hughes, 2003). Similar results were observed in *C. coli* where the terminals of two different flagellin proteins are conserved but the middle regions are antigenically distinct (Harris *et al*., 1987).

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Another form of antigenic variation is flagellin glycosylation. N- and O-linked carbohydrates can be added to flagellin protein to change both its antigenic properties as well as its function (Takeuchi *et al*., 2003). The genes that code for these glycans are usually found adjacent to the *fliC* genes (Ewing *et al*., 2009). Flagellin glycosylation has been found in both Archaea and Bacteria and may play a role in structural assembly, host specificity as well as virulence (Takeuchi *et al*., 2003; Logan, 2006). In *P. syringae* pv *tabaci* flagellin glycosylation plays a role in pathogenicity (Takeuchi *et al*., 2003; Westman *et al*., 2007), swimming motility and acyl homoserine lactone (AHL) production (Westman *et al*., 2007). Plants, mammals and insects all have the ability to detect flagellin (Chinchilla *et al*., 2007; Miao *et al*., 2007) which may be the precedent for flagellar variation and glycosylation as it allows bacteria to avoid the innate immune response of its host.

1.4.2 Twitching motility

Twitching motility is a flagellum-independent form of surface motility (Bahar *et al*., 2009) that allows bacteria to move over a moist surface using retractile, polar type IV pili and results in bacteria rapidly colonising a large surface area (Semmler *et al*., 1999; Liu *et al*., 2001; Li *et al*., 2007). Twitching motility can be defined as the expansion of a colony by the outward migration of bacteria as opposed to colony growth occurring via cell division (Semmler *et al*., 1999; Bahar *et al*., 2009). Under a microscope, twitching colonies have irregular edges, whereas mutant strains have smooth, rounded edges (O'Toole and Kolter, 1998). This is because colonies that exhibit twitching motility form haloes or "spearheads" at their margins (Liu *et al*., 2001; Bahar *et al*., 2009). Using tethered *M. xanthus* cells, it was shown that polar type IV pili adhere to a surface before retracting, pulling the cell in the direction of the pili attachment point. Pili extending from the opposite pole of the cell can then repeat this movement (Sun *et al*., 2000). It was shown in *P. aeruginosa* that the speed of the pilus retraction, and not pilus extension, could be correlated with the movement of the cell, so the cell is in fact being pulled, and not pushed, by the pili (Skerker and Berg, 2001). The same observations were made in *Neisseria gonorrhoeae* (Merz *et al*., 2000).

R. solanacearum twitching motility colonies grew better on the surface of the agar, whereas *P. aeruginosa* grew equally well on either the agar surface or in the interstitial space between the agar and the surface of the Petri dish (Liu *et al*., 2001). It was hypothesised that twitching motility generally occurs best along a smooth surface, i.e. the surface of the agar that set

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against the Petri dish as opposed to the surface that was exposed to air as the agar set (Semmler *et al*., 1999). The outermost edges of twitching motility colonies form "rafts" of 10 – 50 cells that move away from the colony in a clump. Behind the rafts the twitching cells form a lattice-like pattern with structures that are usually only 1 – 5 cells wide. Closer to the colony the lattice starts to solidify as the cells divide and grow to fill in the spaces (Figure 1.3). Type IV pili mutant colonies do not form rafts or lattice-like patterns. It was found that twitching motility in *R. solanacearum* is a quantitative trait that increases as cell density increases (Meng *et al*., 2011).

Type IV pili are also responsible for twitching motility in *R. solanacearum* (Liu *et al*., 2001) where the twitching motility enables the pathogen to spread within its host plant against the vascular flow (Yao and Allen, 2006). Consequently twitching mutants displayed reduced virulence when infecting tomato plants (Yao and Allen, 2006). *Xylella fastidiosa* also uses type IV pili for twitching motility and to move up xylem vessels to colonise upstream vascular regions of the plant (Meng *et al*., 2005). Another example of using twitching motility to move against the flow of a liquid is found in *P. aeruginosa*. It is thought that the ability to migrate upstream allows bacteria to occupy niches that other bacteria cannot reach (Shen *et al*., 2012). In *R. solanacearum* the up-regulation of EPS production during biofilm formation results in the ceasing of twitching motility (Kang *et al*., 2002). Different environmental conditions and the presence or absence of certain chemicals or compounds can affect twitching motility. The increase of AHLs (Chatterjee *et al*., 2008) and calcium, or the decrease of iron concentration greatly increases twitching motility and attachment in *X. fastidiosa* (Zaini *et al*., 2008; Cruz *et al*., 2011).

1.4.2.1 Type IV pilus structure

Type IV pili are found in both Gram-positive and Gram-negative bacteria, as well as some Archaea (Nudleman and Kaiser, 2004; Pohlschroder *et al*., 2011) and they are likely derived from a common ancient ancestor (Imam *et al*., 2011). Type IV pili are the most ubiquitous attachment structures among Gram-negative bacteria and are relatively conserved among bacterial genera (Low *et al*., 1996; Alm and Mattick, 1997). They are retractile, polar and can be present as either single filaments or as tufts of pili (Mattick *et al*., 1996). In *E. coli* K-12, there are 16 type IV pili genes that are clustered at seven different loci (Sauvonnet *et al*., 2000). In general, the components of the type IV pili biogenesis machinery are functionally

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and evolutionarily similar to the components of the type II protein secretion system, also known as the main terminal branch of the general secretion pathway (Figure 1.4) (Nunn, 1999; Thanassi and Hultgren, 2000). The type II secretion system is responsible for secretion of hydrolytic enzymes such as cellulases, pectinases, proteases and toxins (Russel, 1998).

There are two types of type IV pili, namely, type IVA and type IVB pili (Pelicic, 2008). Type IVA pili are relatively common and are usually found associated with attachment, virulence and twitching motility (Pelicic, 2008). The major structural subunit of type IVA pili (PilA) is usually between 150 – 160 amino acids long whereas type IVB pili have either much longer (180 – 200 amino acids) or much shorter (40 – 50 amino acids) subunits. Type IVA pili also have smaller N-terminal signal sequences (less than 10 amino acids) than type IVB pili (15 – 30 amino acids) (Pelicic, 2008). Type IVB pili are thought to play a role in attachment and virulence, although many of them have specialised functions and are found predominantly in enteric pathogens or on mobile genetic elements such as plasmids (Pelicic, 2008; Burdman *et al*., 2011; Burrows, 2012). For example, *V. cholerae* have toxin-coregulated pili (TCP) that are not constitutively expressed but depend on environmental signals (Taylor *et al*., 1987). These pili are up-regulated during toxin production as they serve as phage receptors for a bacteriophage that codes for the proteins that make up the cholera toxin. TCP have also been shown to play a role in attachment (Taylor *et al*., 1987; Novais *et al*., 1998). Another example of type IVB pili are the bundle forming pili (BFP) of *E. coli* (Giron *et al*., 1991). As with TCP, BFP are not constitutively expressed and have been shown to play a role in attachment. BFP assemble into straight fibres that aggregate laterally. They tend to twist, curl and loop over one another, resulting in linking bacterial cells to one another. BFP are predominantly used for cell-to-cell adherence (Giron *et al*., 1991).

Type IV pili are strong, flexible filamentous cell surface structures, about 4 µm long with a diameter of 60 Å (6 nm) (Figure 1.4) (Parge *et al*., 1995; Alm and Mattick, 1997). The engine that drives motion in type IV pili consists of the proteins PilT and PilB (Nudleman and Kaiser, 2004). PilT is an ATPase that mediates the retraction of type IV pili (Burdman *et al*., 2011; Shen *et al*., 2012) whereas PilB is required for pilus extension (Nudleman and Kaiser, 2004). PilB is also involved in the polymerisation of pilin subunits (Burdman *et al*., 2011; Imam *et al*., 2011). These two proteins are located in the cyto- and periplasm and circle the base of the type IV pilin (Nudleman and Kaiser, 2004; Burdman *et al*., 2011). PilM and PilN form an inner membrane complex that aids in protein secretion (Imam *et al*., 2011). The pilus

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extends through the outer membrane via a channel which is made up of the PilQ secretin. Pilin subunits are transported through this channel before assembly and PilQ is thus essential for pilin assembly (Alm and Mattick, 1997; Thanassi and Hultgren, 2000). PilF is associated with PilQ and ensures the correct localisation and oligomerisation of PilQ in the outer membrane (Burrows, 2012). The filament itself consists of repeating subunits of the major pilin protein PilA (Thanassi *et al*., 2012). While the pilin subunits may differ in length and sequence in different species, they always have a conserved N-terminal (approximately 60 amino acids long) which is synthesised with a leader peptide that is cleaved by a prepilin peptidase, PilD or PpdD (Soto and Hultgren, 1999; Nudleman and Kaiser, 2004; Pelicic, 2008). The surface of the pilin is ridged with antigenic regions forming the peaks of the ridges and conserved residues lying within the valleys (Thanassi *et al*., 2012). Adhesins are sometimes found attached at the tip of the type IV pilus, but the major adhesin is found near the major pilin subunit, on the shaft of the pilin (Lee *et al*., 1994). Unlike flagella, type IV pili are not hollow, which implies that they are assembled from the base up, as opposed to polymerisation occurring at their tip (Nudleman and Kaiser, 2004).

PilT plays a role in attachment in *M. xanthus* as, although type IV pili are able to loosely attach to a surface via adhesins, the pili need to be able to retract in order to bring the cell close enough to the surface to form a strong, intimate attachment with the surface (Sun *et al*., 2000). Knock-out mutagenesis of *pilT* in *Acidovorax avenae* resulted in reduced virulence in melons and watermelons (Bahar *et al*., 2009*). P. aeruginosa pilQ*$^{-}$ mutants were unable to synthesise type IV pili and the cells were subsequently unable to undergo twitching motility (Alm and Mattick, 1997). *R. solanacearum pilA*$^{-}$ mutants were unable to form biofilms on polyvinyl chloride (PVC) strips, showed reduced virulence on tomatoes and reduced cell-to-cell aggregation (Liu *et al*., 2001; Kang *et al*., 2002). The *pilA* gene was also knocked-out in *M. xanthus* and *A. avenae* with similar results (Sun *et al*., 2000; Bahar *et al*., 2009). *pilA*$^{-}$, *pilQ*$^{-}$ and *pilT*$^{-}$ mutants were incapable of twitching, and also displayed reduced virulence when infecting tomato plants (Yao and Allen, 2006). In *P. aeruginosa* type IV pili are not constitutively expressed; rather they are expressed only at the outermost edges of colonies where twitching motility is actively taking place. This represents an example of differentiation within the colony (Semmler *et al*., 1999).

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1.4.2.2 The function of type IV pili

Besides twitching motility, type IV pili play an important role in horizontal gene transfer, nutrient acquisition, phage adsorption and multicellular development (Averhoff and Friedrich, 2003; Liu *et al*., 2001; Kang *et al*., 2002; Bahar *et al*., 2009). They are involved in the attachment of cells to surfaces and to one another, thus forming bacterial aggregates (Rojas *et al*., 2002), which often lead to biofilm formation (Soto and Hultgren, 1999; Burdman *et al*., 2011). In the phytosphere, type IV pili have been shown to promote epiphytic fitness and virulence in plant pathogens (Burdman *et al*., 2011; Rojas *et al*., 2002), for example, in *Xanthomonas campestris* pv. *vesicatoria* and *P. syringae* type IV pili increase cell survival on the plant leaf as they play a role in cell aggregation and consequently in UV resistance (Ojanen-Reuhs *et al*., 1997; Roine *et al*., 1998). Pili play a role in early colonisation, before the production of EPS is up-regulated (Kang *et al*., 2002). Surface attachment in plant pathogens is important for successful colonisation and subsequent infections (Alm and Mattick, 1997; Burdman *et al*., 2011).

1.4.3 Swarming motility

Swarming motility occurs on solid or semi-solid surfaces and is a mode of motility used by peritrichously hyperflagellated bacteria (Semmler *et al*., 1999; Herrera *et al*., 2008). Swarming motility is a social form of motility as cells remain in contact with their neighbours. Swarming cells align to one another along their longest side and migrate as a population, forming “rafts” in the process (Fraser and Hughes, 1999). Examples of bacteria that are capable of swarming motility include *Proteus mirabilis* and *Vibrio parahaemolyticus* (Fraser and Hughes, 1999; Kirov, 2003). *Pantoea stewartii* subsp. *stewartii*, which causes Stewart’s vascular wilt in maize, uses swarming motility to form biofilms and colonise xylem vessels. Swarming motility requires the production of stewartan (EPS) and was rendered completely non-motile when a *fliC*$^{-}$ mutant was generated. The master flagella operon, *flhDC*, also regulates swarming motility (Herrera *et al*., 2008).

Swarming, as with twitching motility, allows bacteria to rapidly colonise a surface. Swarming is a form of cell differentiation and is often accompanied by the up-regulation of virulence factors such as urease and metalloprotease (Kirov, 2003). Cell differentiation involves vegetative cells becoming elongated and hyperflagellated (Fraser and Hughes, 1999; Herrera *et al*., 2008). The increased flagellar expression of swarming cells is induced by surfaces with

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high viscosity (Kirov, 2003). To diminish the effect of friction, EPS is produced to increase surface fluid dynamics and to encapsulate the rafts during motility (Fraser and Hughes, 1999; Herrera *et al*., 2008). *P. aeruginosa* uses rhamnolipids to overcome surface tension during swarming motility (Caiazza *et al*., 2005). During early biofilm formation, swarming motility plays a role in helping bacteria to aggregate and form microcolonies. Various environmental factors affect swarming motility, for example, glucose and viscosity increase motility whereas it is inhibited by potassium ions (Herrera *et al*., 2008). Cell density, chemotaxis and surface contact are also important in establishing swarming motility (Fraser and Hughes, 1999; Herrera *et al*., 2008).

1.4.4 Other types of motility

The marine cyanobacterium *Synechococcus* is able to swim without any flagella or pili, merely rotating about its own axis. It has been proposed that *Synechococcus* has a helical, looped track that runs along its surface. Protons driven by the proton motive force move along this track, resulting in a fluid motion next to the track. This leads to a propulsive thrust, which allows the bacteria to move forward in a liquid environment (Ehlers and Oster, 2012).

Other types of motility include self-electrophoresis and expulsion of fluid. Self-electrophoresis occurs when ions are pumped out of the head of the cell and taken in at the tail. This creates an electric field and the fluid in the medium will flow backwards over the cell, consequently moving the cell forward (Pitta and Berg, 1995). Bacteria such as *E. coli* are capable of electrophoretic motility (Lytle *et al*., 1999). Myxobacteria and some cyanobacteria are able to move by drawing in fluids and then expelling them through pores in their surface, a phenomenon known as jet propulsion (Spagnolie and Laugna, 2010).

1.4.5 Biofilms

Bacteria generally occur in two states; planktonic or cellular aggregates. The planktonic state is when bacteria are present as single cells and are unattached to a surface, usually floating or swimming through the environment. The second state occurs when bacterial cells attach both to a surface and to one another to form biofilms (Sternberg *et al*., 1999). Biofilms are complex structures that generally consist of a vast array of microorganisms, held together by an adhesive and protective matrix (Costerton *et al*., 1999). Biofilms are often structured in such a way that microcolonies within the biofilms are separated by water channels. Biofilms protect

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bacteria from harsh environmental conditions by creating a more suitable environment for the cells within the biofilm, and also allow the bacteria to perform functions that would not have been possible in the planktonic state (Morris and Monier, 2003). Most of these functions are as a direct result of quorum sensing. The densely packed structure of the biofilm provides a favourable environment within which to communicate via inducer molecules (Van Houdt *et al.*, 2004). Moreover, biofilms not only affect the physical and chemical environment surrounding the bacterial cells, but also alter their phenotypic behaviour and genetic composition (Morris and Monier, 2003).

In *E. coli*, motility is crucial to the formation of biofilms, whereas chemotaxis-impaired mutants still formed biofilms that were comparable to the wild-type (Pratt and Kolter, 1998). Twitching motility is important in the spread of a biofilm and in the formation of micro-colonies, and also in forming multicellular-layer biofilm structures (O'Toole and Kolter, 1998). Flagella play an important role in biofilm formation as they help the bacterial cell to overcome the repulsive forces existing between the cell and the surface, as well as helping the cell to move along the surface which facilitates biofilm expansion (Pratt and Kolter, 1998; Klausen *et al*., 2003; Malamud *et al*., 2011). Preceding biofilm formation, type I or type IV pili are responsible for the initial attachment to the leaf surface. This is an important step in the development of a disease (Pratt and Kolter, 1998; Kang *et al*., 2002). However, in *P. aeruginosa* it was found that flagella and type IV pili are only important in biofilm development and not in the initial attachment and biofilm formation. Mutants still formed biofilms but with different 3D structures (Klausen *et al*., 2003). Flagellar motility is important in *Listeria monocytogenes* and *Bacillus cereus*, both in attachment and biofilm formation (Lemon *et al*., 2007; Houry *et al*., 2010) and flagella are essential for attachment in *E. coli* (Giron *et al*., 2002). A distinction between the importance of the flagella itself and flagellar motility in attachment and biofilm formation thus needs to be made.

1.5 CONCLUSIONS

Pantoea ananatis is an epiphytic plant pathogen with a broad host range and predominantly causes disease on *Eucalyptus*, maize and onion in South Africa. As *Eucalyptus* and maize are key agronomic crops in South Africa, this plant pathogen warrants further investigation.

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Motility is necessary for the survival and proliferation of plant-associated bacteria as it allows bacterial cells to move towards attractants and away from repellents, as well as to locate, invade and infect their host plant. The most common forms of motility are those that use cellular organelles and the focus of this research project will be swimming and twitching motility. Swimming motility, which is facilitated by flagella, is the fastest means of motility available to bacteria and is used to propel bacteria forward in liquid environments. The flagella are linked to the chemotaxis sensory cascade and the bacteria are thus able to monitor their environmental conditions and adjust their direction of movement accordingly. Flagella also enable bacteria to find and attach to their host. Twitching motility is a relatively rapid means of colonisation and is often used by bacteria to form microcolonies within biofilms, as well as to spread biofilms across a surface. Twitching motility relies on retracting type IV pili. It is likely that once attached to a surface, twitching motility allows the bacteria to spread and possibly gain entry into a plant host.

The recent advances in genome sequencing and availability of genome sequences provide us with the ability to compare the genetic differences in bacteria to shed some light on their effect on varying phenotypes. Here we will use comparative genomic approaches to identify and characterize, *in silico*, the molecular determinants of both swimming and twitching motility. Subsequently, by means of knock-out mutagenesis and phenotypic studies, we will ascertain the role of both twitching and swimming motility in the infection cycle and pathogenesis of *P. ananatis* on onion seedlings.

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FIGURES

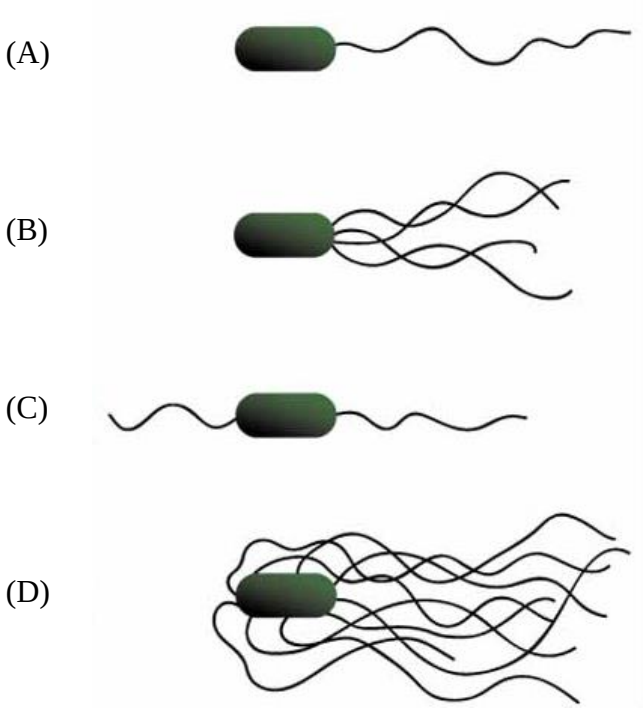

Figure 1.1: Diagram depicting flagellar arrangements on a bacterial cell. (A) Monotrichous, (B) lophotrichous, (C) amphitrichous (D) peritrichous (Image by M. Jones, 2006).

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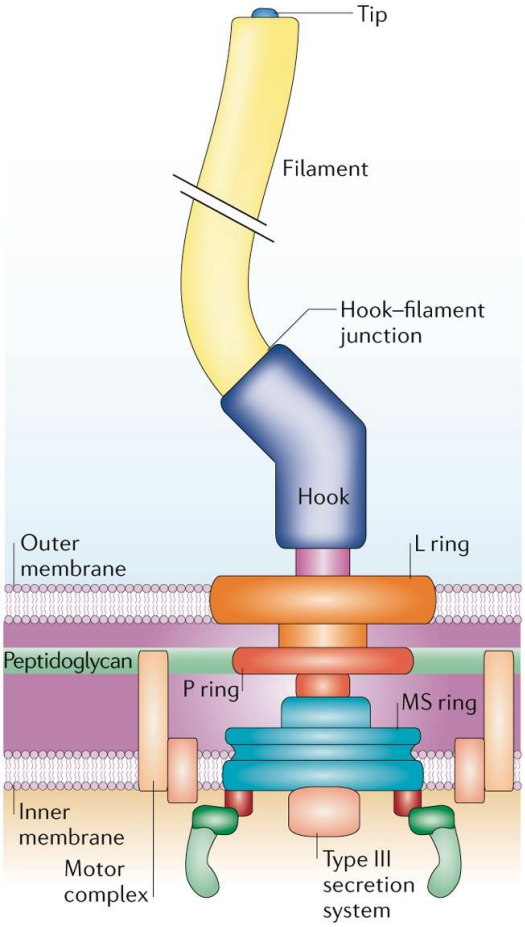

Figure 1.2: Diagram of a bacterial flagellum. The basal body consisting of the MS, P and L rings, is anchored in the membrane and is associated with the motor complex. The hook transfers the torque from the motor to the filament. The whole flagellum also functions as a type III flagellar export pathway (Pallen and Matzke, 2006).

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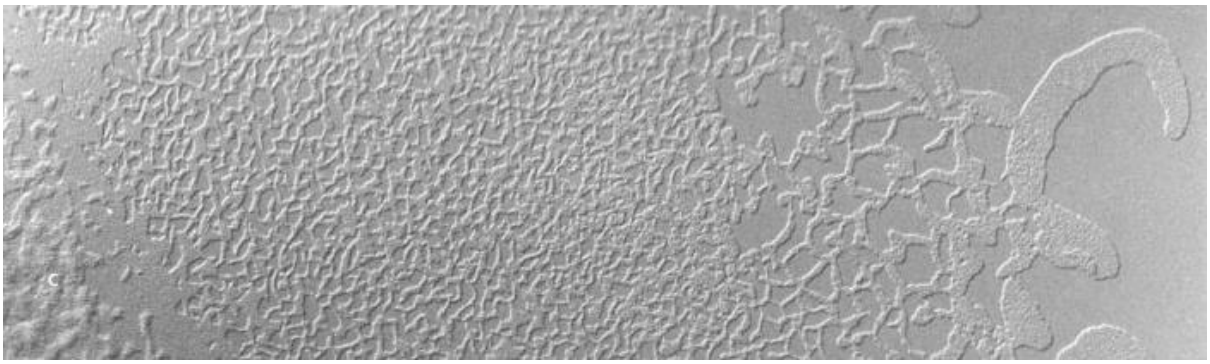

Figure 1.3: Depiction of colony expansion of a twitching colony on agar. On the right-hand side of the image the cells are twitching away from the colony in rafts. Behind the rafts a lattice-like pattern develops and the cells nearest the colony (on the left-hand side) fill up the remaining space through cell division (Semmler *et al*., 1999).

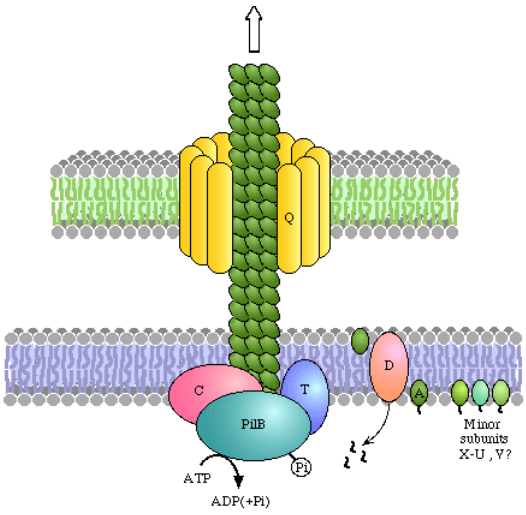

Figure 1.4: Diagram of a type IV pilin (http://www.genome.jp/kegg/pathway/ko/ko03090.html). The filament of the type IV pilin consists of repeating subunits of PilA. The filament extends through the cell membrane via a pore created by PilQ. PilT is involved in twitching motility and enables the pili to retract (Thanassi and Hultgren, 2000).

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CHAPTER 2

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CHAPTER TWO

COMPARATIVE GENOMICS OF *PANTOEA ANANATIS* STRAINS REVEALS MINOR VARIABLILTY IN FLAGELLA AND TYPE IV PILI

2.1 INTRODUCTION

The development of robust next generation sequencing (NGS) technologies has resulted in an explosion of bacterial genome sequences becoming available (Tettelin *et al*., 2008). At present, more than 32,000 bacterial genomes have been sequenced, covering a representative spectrum of genera and species within the Kingdom (www.genomesonline.org). Comparative genomics has revealed extensive intra-species diversity (Tettelin *et al*., 2008; Binnewies *et al*., 2010) which is reflected in the physiology, metabolism, morphology and variety of habitats that bacteria are able to occupy (Binnewies *et al*., 2010). Mining sequenced genomes divulges a lot of information about specific genes. By comparing the genomes of closely related bacteria, the presence, absence or number of specific genes or set of genes which can be correlated to a particular phenotype, or difference in phenotypes among related bacteria, can be determined (Vinatzer and Yan, 2008).

The genus *Pantoea* encompasses a broad range of important phytopathogens, clinical pathogens, and insect endosymbionts with an uncanny ability to adapt and survive in a wide variety of environments (Coutinho and Venter, 2009). As such, there has been increasing interest in this genus and the complete or draft genomes of 48 strains have been sequenced, with another 35 genomes currently being sequenced or targeted for sequencing (www.genomesonline.org). *Pantoea ananatis* is a plant pathogen that is found as both an endophyte and epiphyte on a wide variety of plant hosts and causes disease in, for example, onion (Gitaitis *et al*., 2002; Walcott *et al*., 2002), rice (Watanabe *et al*., 1996), sudan grass (Azad *et al*., 2000) and watermelon (Walcott *et al*., 2003). It has furthermore been isolated from an extensive range of environments that include soil (Coutinho and Venter, 2009), insects (Murrell *et al*., 2003) and sorghum fermentations (Wells *et al*., 2002). *P. ananatis* has also been isolated from humans and identified as a causative agent of bacteraemia (De Baere *et al*., 2004; Van Rostenberghe *et al*., 2006). The ability of *P. ananatis* to cause disease on such a wide variety of hosts and to proliferate in countless different environments suggests

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that individual strains within the *P. ananatis* species have unique genes and traits that allow them to adapt to their environment. These genes are most likely attained through horizontal gene transfer (HGT) from bacteria that co-inhabit their niches.

Motility is advantageous to phytopathogens as it promotes efficient spread, dispersal and general cell fitness. *P. ananatis* is capable of both swimming and twitching motility. Swimming motility is enabled by rotating flagella which are peritrichously situated around the outside of the cell (Shen and Ronald, 2002). The flagellum consists of four main subunits, namely the motor, basal body, hook and filament. The motor is located in the peptidoglycan and not only powers the flagellum to rotate, but also dictates the direction of rotation as it is linked to the chemotaxis cascade (Francis *et al*., 1994; Madigan *et al*., 2003). The motor itself can be divided into the stator (MotAB) and the rotor (FliG, FliM and FliN). While the rotor acts as the switch that changes the direction of the rotation of the flagellum in response to the chemotaxis cascade, the motor establishes the proton motive force that powers the flagellum (Francis *et al*., 1994). The basal body links the motor to the rest of the flagellum and serves to anchor the flagellum within the cell membrane (Kirov, 2003). While the hook transmits the torque generated by the motor to the flagellum filament (Macnab, 2003), it is the rotation of the filament which propels the bacterial cell through its environment (Shen and Ronald, 2002). The hook consists predominantly of FlgE (Macnab, 2003) although hook-associated proteins such as FlgK and FlgL are needed for flagellar synthesis (Ikeda *et al*., 1993; O'Toole and Kolter, 1998). The filament is built with repeating units of flagellin (FliC) (Kirov, 2003) and capped by a capping protein, FliD (Fraser *et al*., 1999; Macnab, 2003).

The genes coding for flagellar biosynthesis and functionality are usually grouped into three genomic clusters in the *Enterobacteriaceae* (Soutourina and Bertin, 2003) and are discussed in greater detail in Section 2.3.1. The expression of the flagellar genes is highly regulated. The master regulator, *flhDC* (Soutourina and Bertin, 2003), activates the expression of the basal body and flagellar export system proteins as well as σ^{28}, which then activates the expression of the subsequent set of proteins. These include the motor, hook, filament and chemotaxis proteins (Soutourina and Bertin, 2003).

The chemotaxis cascade that determines the direction of the rotating flagella is initiated when stimuli bind to the methyl-accepting chemotaxis proteins (MCPs) on the surface of the cell (Mazzag *et al*., 2003). There are four well characterised MCPs in *Escherichia coli*, namely the

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Tar (aspartate), Trg (ribose and galactose), Tsr (serine) and Tap (peptides) receptors (Grebe and Stock 1998; Taylor *et al*., 1999). While the Tar MCPs mediate taxis towards aspartate and maltose, and away from nickel and cobalt, Tsr MCPs bind serine but aid the cell in avoiding leucine, nickel and weak acids (Krikos *et al*., 1985). Trg receptors bind galactose and ribose whereas Tap MCPs mediate taxis towards dipeptides (Grebe and Stock 1998). Strains of *S. enterica* do not have Tap receptors as they have Tcp receptors instead. These mediate taxis toward citrate (Iwama *et al*., 2006). When the stimuli bind to the MCPs they cause a change in the conformation of the MCPs and this consequently excites CheA which initiates the chemotaxis cascade (Kirov, 2003; Macnab, 2003), culminating in CheY being either able or unable to bind to the flagellar motor, depending on whether it was phosphorylated or dephosphorylated (Subramanian and Nott, 2011). When CheY is bound to the motor the flagellum filament rotates in a clockwise direction which causes the cell to tumble (Mazzag *et al*., 2003).

Pili are proteinacious appendages that are anchored in the outer membrane of the bacterial cell, usually with adhesins on their tips. They are filamentous, comprise of helically arranged proteins known as pilin subunits, and are produced by a wide range of bacteria. Most of the filament is composed of a repeating major subunit, although it is usually associated with minor subunits. The minor subunits are generally found at the tip of the appendage, and it is these subunits that make up the adhesins that bind to specific receptors on the host's surface (Soto and Hultgren, 1999). The receptors are usually carbohydrates and the adhesins have high carbohydrate-binding specificity. The binding of these adhesins to their receptor molecules may activate signal transduction pathways, which can have various results such as preparing the bacteria for colonising the host, or alternatively, alerting the host to the presence of the bacteria and initiating host defence mechanisms. Many of these reactions require the activation and expression of new genes (Soto and Hultgren, 1999). While pili attach bacterial cells to the surface and neighbouring cells, attachment is not the only function of pili; they are also responsible for twitching motility and conjugation, and play a role in biofilm formation and pathogenicity (Romantschuk, 1992; Wolfgang *et al*., 2000).

Based on the biochemistry of their biosynthesis, pili can be assembled into four families or groups (Nuccio and Baulmer, 2007). These include the chaperone-usher pathway assembled pili, the general secretion pathway pili such as type IV pili, and pili assembled either via the extracellular nucleation/precipitation pathway or the alternate chaperone pathway (Soto and

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Hultgren, 1999; Nuccio and Baulmer, 2007). Proteins required for the synthesis of pili assembled via the chaperone-usher pathway are secreted into the periplasmic space via the general secretory pathway. They are then bound to chaperone proteins which assists with protein folding. Once bound to the chaperone, the pilin complex travels through the outer membrane via a pore created by the usher protein (Proft and Baker, 2009). Well characterised pili within the chaperone-usher pathway assembled pili include type I-, type II and type III pili (Nuccio and Baulmer, 2007). The components of the type IV pili biogenesis machinery are similar to the type II protein secretion system, also known as the main terminal branch of the general secretion pathway (Thanassi and Hultgren, 2000). Proteins that are secreted via the type II secretion system are synthesised with Sec-dependent signal sequences at their N terminals. The greatest difference between this protein secretion system and the Sec system is that the Sec system translocates unfolded proteins, whereas the type II secretion system translocates correctly folded proteins (Russel, 1998). The major subunit of type IV pili has a leader peptide that has to be cleaved from the subunit for maturation. This is done by an inner membrane signal peptidase (Soto and Hultgren, 1999). Curli are assembled via the extracellular nucleation/precipitation pathway and mediate attachment to human tissues (Soto and Hultgren, 1999). They are assembled extracellularly where the secreted major subunit interacts with the nucleator, a protein anchored in the outer membrane. This results in conformational changes which activates the polymerisation of the major subunit (Wu and Fives-Taylor, 2001). An example of pili assembled via the alternate chaperone pathway are the CS1 pili which are human enterotoxigenic pili (Nuccio and Baulmer, 2007). The chaperones employed in this pathway are distinct from those in the chaperone-usher pathway (Soto and Hultgren, 1999).

Type IV pili enable bacterial cells to move across a surface using twitching motility as they are capable of retraction. The type IV pilus base is situated in the membrane alongside the proteins that provide the energy for retraction (PilB and PilT) (Nudleman and Kaiser, 2004; Burdman *et al*., 2011; Imam *et al*., 2011). HofQ forms a secretin through which the pilus is able to extend through the membrane (Thanassi and Hultgren, 2000) while PilF is found in association with HofQ, ensuring the correct placement of HofQ in the membrane (Burrows, 2012). The filament of the pilus is predominantly made up of PilA but also includes adhesins that aid in attachment (Lee *et al*., 1994). The N-terminal of the PilA has a leader peptide

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which is cleaved and methylated by PilD. This is a necessary step for the secretion of the mature pilin (LaPointe and Taylor, 2000; Pelicic, 2008).

Four complete and nine draft genomes of *P. ananatis* strains, isolated from diverse ecological sources, have been sequenced. Here, by means of comparative genomic approaches we have characterised the genetic loci for flagellum biosynthesis and motility as well as the type IV pili loci of *P. ananatis*. In order to obtain an accurate picture of these genetic loci, we have focused only on those *P. ananatis* strains for which complete genome sequences are available, and compared these to the orthologous loci in other members of the genus *Pantoea* and related *Enterobacteriaceae*. As these loci are important for the biosynthesis and functioning of both flagella and type IV pili, we observed that they are highly conserved both in terms of nucleotide sequence and synteny. Exceptions are the presence of multiple copies of the flagellin (*fliC*) genes in *P. ananatis* and the presence of genes encoding a type IVB pilus restricted to certain *P. ananatis* strains. Their potential roles in the distinct biology of *P. ananatis* are discussed.

2.2 MATERIALS AND METHODS

2.2.1 Identification of the *P. ananatis* flagellar and type IV pili biosynthetic loci

The complete genomes of four *P. ananatis* strains have been sequenced (Table 1). The flagellum and type IV pili biosynthetic loci were identified in the *P. ananatis* genomes using localized tBlastN analyses against the *E. coli* flagellar and type IV pili biosynthetic proteins. The full biosynthetic loci were identified and extracted from the genome genbank files. To standardise the protein coding sequence (CDS) datasets for the loci, the nucleotide sequences for each of the loci were submitted to the FgenesB open reading frame prediction tool (www.softberry.com). Similarly, the complete flagellum and type IV pili biosynthetic and CDS sets were extracted from the genomes of the enterobacterial comparators *E. coli* K-12 MG1655 (NC_000913/U00096), *Salmonella enterica* serovar Typhimurium LT2 (NC_003197/NC_003277), *Erwinia amylovora* CFBP1430 (FN434113-4114) and the complete genomes of *Pantoea vagans* C9-1 (NC_014258/NC_14561-4563) and *Pantoea* sp. At-9b (NC_014837-4842). The genomic locations of each of the *P. ananatis* flagellar and type IV pilus biosynthetic loci were determined, with localised tBlastN analyses against the

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complete genome nucleotide sequences, using Bioedit v 7.1.3.0 (Hall, 1999) and mapped onto the *P. ananatis* genomes using CGView (Grant and Stothard, 2008).

- **Comparative analyses of the *P. ananatis* flagellar and type IV pili biosynthetic proteins**

The CDS sets for the different *P. ananatis* biosynthetic loci and those in the enterobacterial comparators were compared using the reciprocal best BlastP hit approach (RBBH) (Moreno-Hagelsieb and Latimer, 2008), through localized BlastP analyses with Bioedit (Hall, 1999). The sum of the total number of identities in the dataset was divided by the sum of the total length of the alignments to calculate the average amino acid identities. Orthology was assumed for those CDSs sharing over 70% amino acid identity over 70% of the sequence lengths. By this means those flagellum and type IV pilus biosynthetic proteins core to the *P. ananatis* and enterobacterial comparators were identified.

- **Phylogenetic analyses**

Phylogenies were constructed using the amino acid sequences of the core flagellar and type IV pilus biosynthetic proteins. The amino acid sequences of the core proteins were concatenated. Similarly, the amino acid sequences for the house-keeping markers AtpD (ATP synthase F1, β subunit), GyrB (gyrase, subunit B), InfB (translation initiation factor IF-2) and RpoB (RNA polymerase, β subunit) were concatenated. The concatenated sequences were aligned using the MAFFT server (version 7) with the BLOSUM62 scoring matrix option, and the FFT-NS progressive alignment option (Katoh *et al*., 2002; Katoh and Standley, 2013). From the alignments, neighbour-joining trees were constructed, with the option Poisson correction, complete gap deletion and bootstrap analyses (n = 1,000), using Mega 6 (Tamura et al. 2013).

2.3 RESULTS AND DISCUSSION

2.3.1 Comparative genomic analyses of the flagellar biosynthetic loci in sequenced *P. ananatis* and enterobacterial comparator strains

In the enterobacteria, approximately 50 genes are involved in flagellar synthesis and functioning. In *E. coli* K-12 MG1655 and *Salmonella enterica* serovar Typhimurium LT2, 50 and 51 genes are involved in flagellum biosynthesis, respectively, and these are organized into

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three chromosomal clusters (I to III) (Soutourina and Bertin, 2003). Cluster I comprises predominantly flagellar structural genes whereas Cluster II includes those genes needed for assembly, chemotaxis and the flagellar motor. Cluster III contains both structural and assembly genes and has since been subdivided into two clusters IIIa and IIIb, separated by 2.7 kb in *E. coli* and 0.8 kb in *S. enterica* (Raha *et al*., 1993; Soutourina and Bertin, 2003)

In the four *P. ananatis* strains analysed, the flagellum biosynthetic and functional genes are clustered in seven loci (FI – FVII), five of which are mapped on the chromosome, while two are localized on the LPP-1 plasmid present in all *P. ananatis* strains (De Maayer *et al*., 2012a) (Figure 2.1 and Figure 2.2). Between 61 and 73 proteins with a predicted role in the biosynthesis and functioning of the flagellum are encoded in these loci. Of the 50 and 51 predicted flagellar genes of *E. coli* K-12 and *Salmonella enterica* serovar Typhimurium LT2, 47 of each are also encoded in the *P. ananatis* flagellar loci, respectively. *P. ananatis* loci FI and FIV (Figure 2.4 and 2.7), show extensive sequence homology and synteny to the *E. coli* K-12 and *S. enterica* flagellar gene clusters I and IIIb respectively. Loci FI and FIV predominantly carry the basal body and hook structural genes. FlgM, which regulates the transcription of late flagellar gene products i.e. the flagellar filament and several hook, basal body and motor proteins, is also found at locus FI (Figure 2.4). Locus II, which shows synteny to the *E. coli* K-12 and *S. enterica* flagellar gene cluster II, codes for the chemotaxis and motor proteins, MotAB. A non-orthologous region is present within FII (Figure 2.5), which is, for the remainder, well conserved and this variable region encodes distinct MCPs. Two *tsr* genes encoding serine-specific MCPs are found between the *cheR* and *cheW* genes in the four *P. ananatis* strains and *E. amylovora* CFBP1430, while three are present in the same location in *P. vagans* C9-1. By contrast, the same region in *Pantoea* sp. At-9b encodes one serine- (Tsr) and two aspartate-binding MCPs (Tar). *E. coli* K-12 MG1655 encodes both Tar and Tap MCPs while *S. enterica* serovar Typhimurium only has a *tar* gene. The MCPs encoded in FII can therefore drive the directional change of flagellar motility in *P. ananatis* in response to the stimuli present in the environment. Unlike the other strains included in this analysis, the FII locus in *Pantoea* sp. At-9b does not encode FlhBAE, and these are encoded in a distinct cluster elsewhere on the chromosome. While *flhDC* is the master flagella regulator (Lee and Harshey, 2012), the *flhBAE* operon plays an important role in the flagellar type III export system (Stafford and Hughes, 2007). The FIII genes encoding flagellar structural proteins (Figure 2.3) are well conserved and syntenous with those of the cluster IIIa genes of *E. coli* and *S. enterica*. However, a non-conserved region, ranging in size from 9 to

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22.7 kb, can be observed between *fliA* and the flagellin gene *fliC* (Figure 2.6). This region is unique among the compared strains to *P. ananatis*. Genes within this non-conserved region include *rmlD*, *rmlC* and *rmlA* which code for orthologous enzymes that play a role in the synthesis of TDP-L-rhamnose. Similarly, genes encoding glycosyltransferases can be observed in this location in all the *Pantoea* strains, however, they are absent from *E. amylovora* CFBP1430, *S. enterica* serovar Typhimurium LT2 and *E. coli* K-12 MG1655 (Figure 2.6). It is likely that the genes in this region code for a glycan as well as the enzymes required for the glycosylation of the flagellin (FliC) protein. Similarly, flagellin glycosylation has been observed in a number of phytopathogenic bacteria, including *Pseudomonas syringae*, *Dickeya dadantii*, *Pectobacterium carotovorum* and *Xanthomonas campestris* (Ichinose *et al.*, 2013) and has been shown to play a role in virulence in *P. syringae* serovars and *X. campestris* (Takeuchi *et al.*, 2003; Ichinose *et al.*, 2013; Logan *et al.*, 2006). As flagellin glycosylation in *P. ananatis* is the subject of another student's research project in our group, it falls out of the scope of this project and will not be discussed further. Loci FV – FVII are only found in the *P. ananatis* strains as the other strains included in this study only have one copy of the flagellin gene (Fgures 2.8 – 2.10).

- **The genomes of *P. ananatis* strains encode multiple copies of the major flagellum structural protein FliC**

Of note in the *P. ananatis* FIII region is the presence of multiple orthologous copies of the *fliC* gene, with three copies found in *P. ananatis* LMG5342, while the remaining three strains have four copies each (Figure 2.6). Given the highly conserved sequence of the flagellin genes, it is possible that *P. ananatis* LMG5342 likewise contains a fourth copy within this locus, but may have been mis-assembled. An additional *fliC* ortholog is found in the chromosomal locus FV (Figure 2.8). Furthermore, two additional *fliC* orthologs are encoded on the LPP-1 plasmid. Thus seven copies of FliC are encoded on the genomes of *P. ananatis* AJ13355, LMG20103 and PA13, while six copies are encoded on the genome of *P. ananatis* LMG5342. By contrast, only a single copy is encoded on the genomes of the comparator strains, including strains of other species in the genus. However, given the high level of sequence homology of the *fliC* genes, it is possible that mis-assemblies may have resulted in the genomes of the comparator strains, and that multiple copies of *fliC* may yet occur in one or more of these strains.

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The additional copies of the *fliC* gene in *P. ananatis* may be indicative of phase variation, as has been found in other bacteria such as pathogenic strains of *E. coli* (Liu *et al*., 2012), *Campylobacter jejuni* and *Campylobacter coli* (Harris *et al*., 1987; Park *et al*., 2000). Phase variation of surface structures such as flagella, fimbriae and EPS is a reversible process that allows bacteria to avoid being detected by the host and eliciting a defence response, as well as enabling the bacteria to adapt to their environment (Hallet, 2001; Liu *et al*., 2012; Yang *et al*., 2012). Flagellin is a major surface antigen in Gram-negative bacteria and has a highly conserved 22 amino acid sequence in the N-terminus of flagellin (flg22) which activates host plant defences (Bonifield and Hughes, 2003; Zipfel *et al*., 2004; Liu *et al*., 2012). Plants are highly sensitive to flg22 (Felix *et al*., 1999). However, in several bacterial species such as *Edwardsiella ictaluri* (Panangala *et al*., 2009), the N- and C-terminals are hydrophobic as opposed to hydrophilic, resulting in the exposed central region of the protein determining the antigenic properties of the flagellin (Panangala *et al*., 2009). Both the flagellin N- and C-terminals are generally well conserved as they play a role in export and assembly (Malapaka *et al*., 2007). The antigenic variability within the alignment of the FliC proteins of *P. ananatis* showed that similarly the N- and C-terminals are conserved, while the central portion, which represents the surface-exposed antigenic region, is highly variable (Figure 2.11).

2.3.2 Flagellar motility in *P. ananatis* is an ancient and vertically maintained trait

The flagellar protein datasets, consisting of 47 core proteins conserved in all of the strains included in this study, were pair-wise compared using BlastP. The average amino acid identity between the flagellar proteins of the *P. ananatis* strains is 99.5% whereas the average amino acid identity between the *P. ananatis* and comparator *Pantoea* strains (*P. vagans* C9-1 and *Pantoea* sp. At-9b) is 89.58% (Table 2.2). Relatively high levels of sequence identity were also observed between *P. ananatis* and the more distant enterobacterial relatives *E. coli* K-12 MG1655 (68.00%) and *S. enterica* serovar Typhimurium LT2 (68.84%).

The evolutionary history of the flagellum biosynthetic proteins was inferred on the basis of alignments of the 47 core proteins. The amino acid sequences of the core proteins were concatenated and aligned using MAFFT server (version 7) and a neighbour-joining tree was constructed (Figure 2.12). The total protein alignment consisted of 18,433 amino acid positions. The phylogeny of the flagellar genes was compared to a phylogeny constructed on the basis of an alignment of the amino acid sequences of the house-keeping markers AtpD,

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GyrB, InfB and RpoB. The data set for the house-keeping proteins consisted of 4,334 amino acids positions. While some difference could be observed in the clustering of the *P. ananatis* strains between the house-keeping and flagellar biosynthetic protein trees (Figure 2.12), the overall topologies of the two phylogenies are well-conserved. The similar topologies suggest that, as is the case for the house-keeping proteins which are required for vital cellular functions, flagellum biosynthesis and flagellar motility are ancestral traits whose incorporation in the genome pre-dates speciation events within the family *Enterobacteriaceae*. Thus, the flagellum biosynthetic and functional loci appear to be vertically maintained within *P. ananatis*, being passed from one generation to the next.

2.3.3 Eight distinct pili are encoded on the genomes of sequenced *P. ananatis* strains

Analysis of the complete annotated genomes of the four compared *P. ananatis* strains showed that eight putative pili belonging to two fimbrial families are encoded on their genomes (Table 2.3). While the genomes of the four compared *P. ananatis* strains do not code for any pili assembled via the alternate chaperone or extracellular nucleation/precipitation pathways, all four strains code for pili assembled via the chaperone-usher and general secretion pathways. The chaperone-usher assembled pili of the four *P. ananatis* strains include five distinct type I pili, namely the Stb-, Csu- and P pili, mannose resistant frimbriae (MRF), and an unclassified type I pilus. The Stb- and Csu pili and the MRF are present in all four *P. ananatis* strains whereas P pili are only found in strain LMG5342. An additional unclassified type I pilus is only encoded on the AJ13355 chromosome. An operon coding for orthologs of proteins involved in the synthesis of a type III pilus, is likewise present in all four *P. ananatis* strains. Type IV pili belong to the family of pili assembled via the general secretion pathway. Whereas type IVA pili are prevalent in all the *P. ananatis* strains, type IVB pili are only present in strains LMG5342 and PA13. These pili are briefly discussed below with the focus on type IV pili.

2.3.3.1 Chaperone-usher pathway assembled pili

Well-known pili that belong to the family of chaperone-usher pathway assembled pili include type I- (Proft and Baker, 2009) and type III pili (Nuccio and Baumler, 2007; Johnson *et al*., 2011). The dominant function of these pili is attachment to a variety of surfaces and hosts (Thanassi *et al*., 2012).

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Type I pili bind to both secreted and cell-bound mannosylated glycoproteins or, more specifically, mannose-oligosaccharides (Soto and Hultgren, 1999; Sauer *et al.*, 2000). As type I pili typically bind to mannosylated glycoproteins, adhesion mediated by type I pili can usually be inhibited by the presence of free α-methyl-D-mannose. Type I pili are capable of unspecific hydrophobic attachment as well (Haahtela *et al*., 1985). In *E. coli* type I pili may stimulate apoptosis in the cells of the host plant, which is designed to retard the progression of the infection in the tissue (Mulvey *et al*., 1998). Type I pili have also been observed to aid in biofilm formation and invasion of host cells (Klemm *et al*., 2006). Several type I pili, including Stb-, Csu- and P pili, as well as MRF, have been identified and classified in the *Enterobacteriaceae*. Between three and four type I pili are encoded on the genomes of the compared *P. ananatis* strains (Table 2.3).

Five genes, *stbABCDE*, are present on the genomes of all four compared *P. ananatis* strains (AJ13355: PAJ_0865-0869; LMG20103: PANA_1520-1524; LMG5342: PANA5342_2705-2709; PA13: PAGR_g2621-2625) and encode orthologs of the *S. enterica* pili biosynthetic proteins. The encoded proteins share 59.90% average amino acid identity with the StbABCDE proteins of *S. enterica* serovar Typhimurium LT2 (AAL19294.1 – AAL19290.1). The Stb pilus has been shown to allow *S. enterica* to be stably maintained in mice and chickens (Weening *et al*., 2005; Clayton *et al*., 2008). It is therefore possible that Stb fimbriae aid *P. ananatis* in attaching to eukaryotic tissues. In the human pathogen *Yersinia pestis* and the environmental bacterium *Pseudomonas putida*, Csu fimbriae play a role in attachment to biotic surfaces. Csu pili are encoded for by the five genes; *csuABCDE* (Tomaras *et al*., 2003), which are present on the genomes of all four *P. ananatis* strains (AJ13355: PAJ_2575-2579; LMG20103: PANA_3330–3334; LMG5342: PANA5342_0728-0732; PA13: PAGR_g0744, PAGR_g0746-0749). The orthologs of the Csu pilin proteins in the four *P. ananatis* strains share 44.23% average amino acid identity with the Csu proteins in *P. putida* KT2440 (AAN67972.1 – AAN67976.1).

Mannose-resistant fimbriae may contribute to the pathogenicity of several bacteria, including *Proteus mirabilis* (Bahrani and Mobley, 1994) and *Photorhabdus temperata* (Meslet-Cladiere *et al*., 2004), where they play a role in swarming motility and attachment or colonisation to mammalian and insect cells, respectively. These fimbriae are known as mannose-resistant fimbriae as they have the ability to agglutinate human erythrocytes in the presence of D-mannose, and bacterial adhesion to mammalian cells via the MRF is not inhibited by the

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presence of D-mannose (Hull *et al*., 1981; Clegg and Pierce, 1983). In *P. temperata*, the MRF are encoded by the *mrp* operon, which includes 10 genes, with gene *mrfA* encoding the major subunit (Meslet-Cladiere *et al*., 2004). In *Serratia entomophila* the MRF genes are organised in the *sefA-J* operon, with the major subunit being SefA (Hurst *et al*., 2003). MRF pili are encoded on the genomes of all four *P. ananatis* strains (AJ13355: PAJ_2006-2015; LMG20103: PANA_2716–2717, PANA_2721–2726; LMG5342: PANA5342_3203-3210; PA13: PAGR-g1299-1304, PAGR_g1307-1310) and these biosynthetic proteins share greatest orthology with the Sef pili in *Serratia entomophila* (Hurst *et al*., 2003). The ten *P. ananatis* MRF pilin proteins share an average amino acid identity of 46.54% with the MRF proteins on the *S. entomophila* plasmid pADAP (AAR13147.1 – AAR13156.1). Similarly to what has been observed in *P. temperata*, it is possible that the *P. ananatis* MRF pili may play a role in attachment to insect hosts, which have been demonstrated to serve as vectors for this bacterium (Takahashi *et al*., 1995; Watanabe *et al*., 1996; Gitaitis *et al*., 2003).

Another type of type I pili, P pili, usually bind to Galα1 – Gal4β - containing glycolipids, or to the terminal sugars of glycolipids on human tissue surfaces (Leffler and Svanborg-Eden, 1980). The genes that encode the P pili are found in the *papA-K* operon (Wullt *et al*., 2002). P pili are composite fibres, consisting of flexible fibrillae joined to pilus rods (Kuehn *et al*., 1992). The tip consists predominantly of PapE, but other proteins, such as the adhesin PapG and adaptor proteins PapF and PapK, are located at the tip as well. P pili play a role in attachment to human tissues (Mulvey *et al*., 1998). Of the *P. ananatis* strains included in this study, P pili were only found in strain LMG5342. Orthologs of the P pili proteins (PANA5342_1331-1338) share 41.93% average amino acid identity with the P pili biosynthetic proteins in *E. coli* CFT073 (AAN82031.4 – AAN82041.1). *P. ananatis* LMG5342 is a clinical strain and was isolated from a human wound (de Maayer *et al*., 2012b). It may thus be that P pili allow strain LMG5342 to attach to human hosts whilst most other *P. ananatis* strains are isolated from plants or environmental samples.

An additional unclassified type I pilus is encoded on the genome of *P. ananatis* AJ13355. Orthologs of the major pilin subunit, FimA (PAJ_0947), share 53.93% amino acid identity with the FimA subunit (AIZ92916.1) of an uncharacterised type I pilus in *E. coli* K-12 MG1655. FimF (PAJ_0943) and FimG (PAJ_0942) make up the tip of the pilus and share 50.00% and 52.14% amino acid identity, respectively, with the corresponding proteins (AIZ92912.1 and AIZ92911.1) in *E. coli* K-12 MG1655 (Jones *et al*., 1995; Soto and

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Hultgren, 1999). The mannose-sensitive adhesin, FimH (PAJ_0941) shares 29.21% amino acid identity with its *E. coli* K-12 MG1655 paralog (AIZ92910.1). FimC (PAJ_945) and FimD (PAJ_944), the chaperone and usher proteins, share 51.82% and 54.05% amino acid identity with the *E. coli* K-12 MG1655 chaperone and usher proteins (AIZ92914.1 and AIZ92913.1, respectively). FimI (PAJ_0946), which is an ortholog of the *E. coli* K-12 MG1655 type I pili biogenesis protein (AIZ92915.1), shares 46.05% amino acid identity.

Type III pili mediate attachment in several *Enterobacteriaceae*, including attachment of a *Klebsiella* spp. to plant roots (Korhonen *et al*., 1987; Ong *et al.*, 2008). They are encoded for by six genes, *mrkEABCDF*, with the encoded protein MrkA being the major pilin subunit (Huang *et al*., 2009). Type III pili also play a role in biofilm formation on several different surfaces (Ong *et al*., 2008; Huang *et al*., 2009). Mrk-like fimbriae are encoded on the genomes of all four compared *P. ananatis* strains (AJ13355: PAJ_3758-3762; LMG20103: PANA_0623–0627; LMG5342: PANA5342_3689-3693; PAGR_g3571-3575) and the MrkEABCDF proteins in these strains share 31.74 % average amino acid identity with the Mrk proteins (CD012585.1 – CD012590.1) in *Klebsiella pneumoniae* Kp52.145 (Allen *et al*., 1991).

2.3.3.2 Pili assembled via the general secretion pathway

Pili that are assembled via the general secretion pathway include the type IV pili (Soto and Hultgren, 1999). These pili are involved in motility, namely twitching motility, conjugation and general cell fitness (Kang *et al*., 2002; Rojas *et al*., 2002) They can be divided into two groups, namely type IVA and type IVB pili, depending on the modification of their pilin subunits as a leader peptide is removed from the pilins by a prepilin peptidase before oligomerisation. Type IVA pili usually have a shorter leader peptide than type IVB pili (Roux *et al*., 2012). As the type IVA pili are dealt with in greater detail below, only the type IVB pili will be discussed here.

The genes encoding type IVB pili are clustered into operons and are most likely to have been acquired by HGT (Roux *et al*., 2012). There are seven known type IVB pili expressed in Gram negative bacteria, including BFP (bundle-forming pili), TCP (toxin-co-regulated pili), R64 thin pili, Flp (fibril-associated protein) pili, Cpa pili, Lng (longus) pili and Cof pili (Roux *et al*., 2012). *P. ananatis* LMG5342 and PA13 both have a predicted type IVB pilus locus,

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comprising the genes *pilL-pilV* in the type IVB locus (Figure 2.14), which is absent from the other two compared strains (AJ13355 and LMG20103). This *pil* locus is flanked upstream by genes encoding the conjugal transfer and coupling proteins, TraG, TraC, TraD and TraW and the transglycosylase TraL (de Maayer *et al*., 2015). *pilL-pilV* and its flanking genes are located on an integrative and conjugative element, ICE*Pan*, which is relatively prevalent among *P. ananatis* strains, and plays a role in the transfer of cargo genes coding for protein products with potential roles in stress response and antibiosis (De Maayer *et al.*, 2015). It is thus highly likely that the type IVB pilus in *P. ananatis* LMG5342 and PA13 forms part of the conjugative machinery for the ICE*Pan* element.

2.3.3.3 Comparative analyses of type IV pili biosynthetic loci in sequenced *P. ananatis* and enterobacterial comparator strains

Type IVA pili are encoded on the genomes of all four sequenced *P. ananatis* strains and are very well conserved (Figures 2.15 - 2.21). Type IVA pili are commonly known as type IV pili. The type IVA pili in *P. ananatis* will thus henceforth be referred to as type IV pili. The 16 genes encoding the type IV pilus proteins in *P. ananatis* are scattered across the genome at eight distinct loci (Figure 2.13). This is comparable to other members of *Enterobacteriaceae*, for example, *E. coli* K-12 has 16 type IV pili genes that are located at seven different loci on its genome (Sauvonnet *et al*., 2000). Locus I is well conserved and encodes the major pilin subunit PilA, as well as PilB and PilC. PilB not only polymerises pilin subunits (Burdman *et al*., 2011), but also works alongside PilT, enabling twitching motility (Nudleman and Kaiser, 2004). PilC plays a role in type IV pilus assembly (Karuppiah *et al*., 2010). Locus II only encodes PilF which plays a role in type IV pili assembly, although this protein is absent from *S. enterica* serovar Typhimurium LT2 and *E. coli* K-12 MG1655. PilF is a pilotin which aids the biogenesis of the secretin (HofQ) in the outer membrane (Korotkov and Hol, 2013). It may be that these two strains encode non-orthologous proteins that play a similar role to PilF. For example, while *E. coli* K-12 MG1655 does not carry a gene encoding PilF, it does code for the putative pilotin YghG (Korotkov and Hol, 2013). Loci III, IV and VI are well conserved amongst all the strains included in the comparative study. While locus III encodes prepilin peptidases that are required for processing the major pilin subunit (LaPointe and Taylor, 2000), locus IV plays a dominant role in motility as it encodes the twitching motility ATPase, PilT. Locus VI encodes type IV pili biogenesis and assembly proteins. Similar to locus III, locus V encodes a prepilin peptidase, PilD. PVII is unique among the compared strains to the four *P. ananatis* strains. PilJ, which is encoded in locus VII, forms part of the

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pilus biogenesis machinery and is required for full pilus assembly. PilJ may also play a role in pilus extension (DeLange *et al*., 2007).

2.3.3.4 Type IV pili are well conserved in *P. ananatis*

The type IV pili core gene datasets consists of thirteen proteins that were compared using BlastP. The average amino acid identity between the thirteen core proteins among the *P. ananatis* strains is 97.17%, whereas the average amino acid identity between all of the compared *Pantoea* strains (including *P. vagans* C9-1 and *Pantoea* sp. At-9b) is 80.18% (Table 2.4). A neighbour-joining tree was constructed by concatenating the core amino acid sequences of the type IV pili proteins using the MAFFT server (Figure 2.22). A total of 6,223 amino acid positions were used to infer the evolutionary relationship of the type IV pili core genes. This was compared to the topology of the house-keeping markers AtpD, GyrB, InfB and RpoB and it was observed that, while the topology of the trees was similar, differences in the clustering of the *P. ananatis* strains were evident. Type IV pili are essential to general cell fitness and virulence and are thus well conserved and transferred vertically from one generation to the next.

2.4 CONCLUSIONS

Both flagella and type IV pili are ubiquitous amongst plant pathogenic bacteria and play a central role in their proliferation and pathogenicity. It is thus not surprising that both motility organelles are well conserved in the four sequenced *P. ananatis* strains compared in this study. While there are only minor discrepancies in the genomic organisation of the flagellar genes, it was noted that *P. ananatis* has additional copies of the *fliC* gene while the comparator strains have only one. The *fliC* genes are located in four different loci across the genome, with two of the loci being on the LPP-1 plasmid. This suggests that *P. ananatis* is able to undergo phase variation to prevent its flagella from triggering a host immune response during infection. *P. ananatis* has eight different pili that predominantly aid in attachment to various different hosts and surfaces. The type IV pili are well conserved and share a similar phylogeny to the house-keeping genes. Strains PA13 and LMG5342 have an additional type IVB pilus that plays a conjugative role.

These results provide additional information on how *P. ananatis* is able to infect such a wide variety of hosts. While motility is advantageous to any plant pathogen as a means of locating

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its host, colonisation, infection and spread, the ability to avoid the innate immune system of the host and to attach to a variety of abiotic and biotic surfaces, greatly increases the pathogenic prowess of *P. ananatis*. The conserved nature of the flagella and type IV pili led to the hypothesis that both swimming and twitching motility play a dominant role in the survival and colonisation of *P. ananatis* on its host plants. The ensuing chapters therefore focus on the mutation of genes crucial to the structure and functionality of the flagella and type IV pili, and the resulting phenotypic changes in *P. ananatis*.

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FIGURES

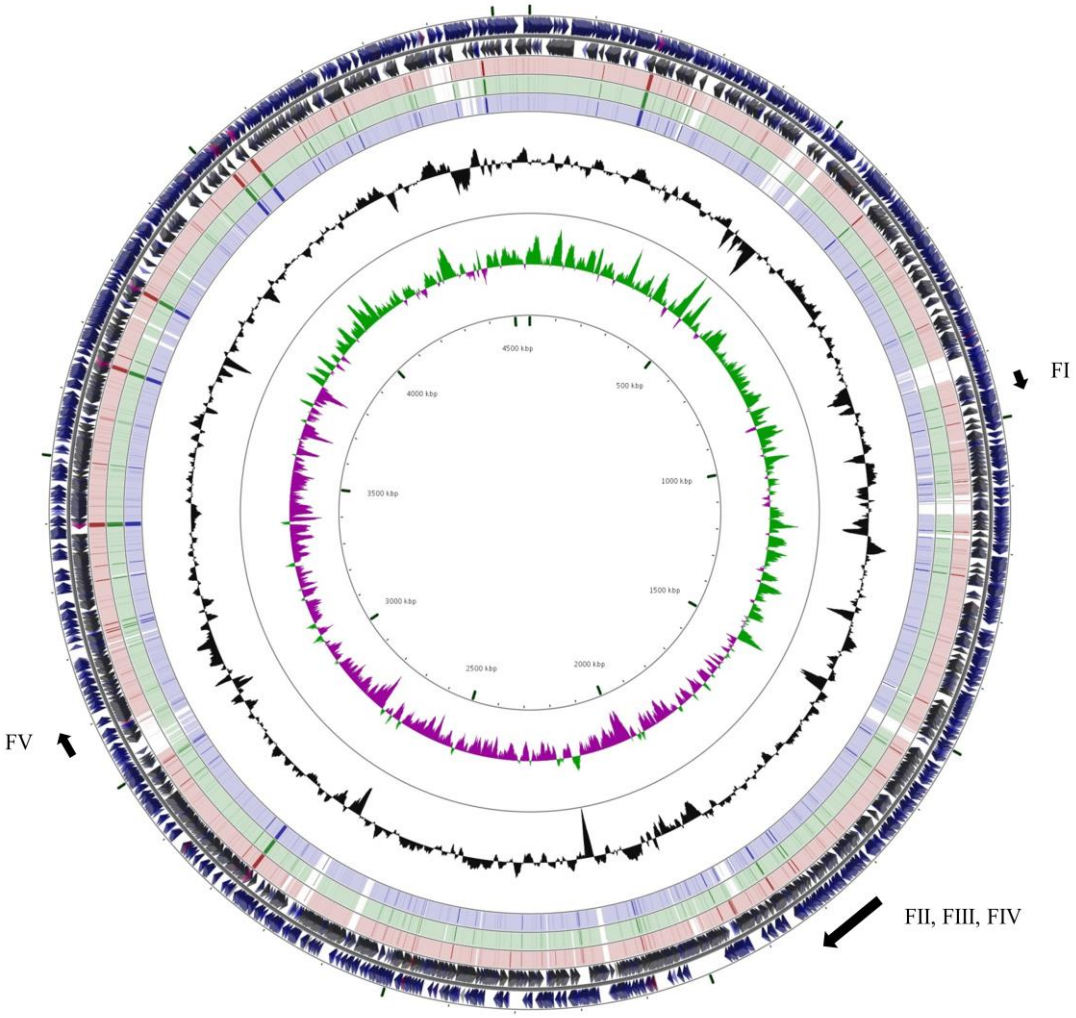

Figure 2.1: Genome comparison diagram of the chromosomes in *P. ananatis*. The flagellum biosynthetic and functional genes are clustered into five loci on the chromosome, indicated on the map as FI, FII, FIII, FIV and FV. The two outer rings depict the CDSs of *P. ananatis* AJ13355 in both a forward and reverse orientation whereas the red, green and blue rings represent *P. ananatis* LMG20103, PA13 and LMG5342, respectively. Flagellum loci FI and FIV include many of the structural and assembly genes whereas FII consists of the chemotaxis and motor protein encoding genes, including *motA* and *motB*. *fliC*1-4 are located in FIII and FV is *fliC*5.

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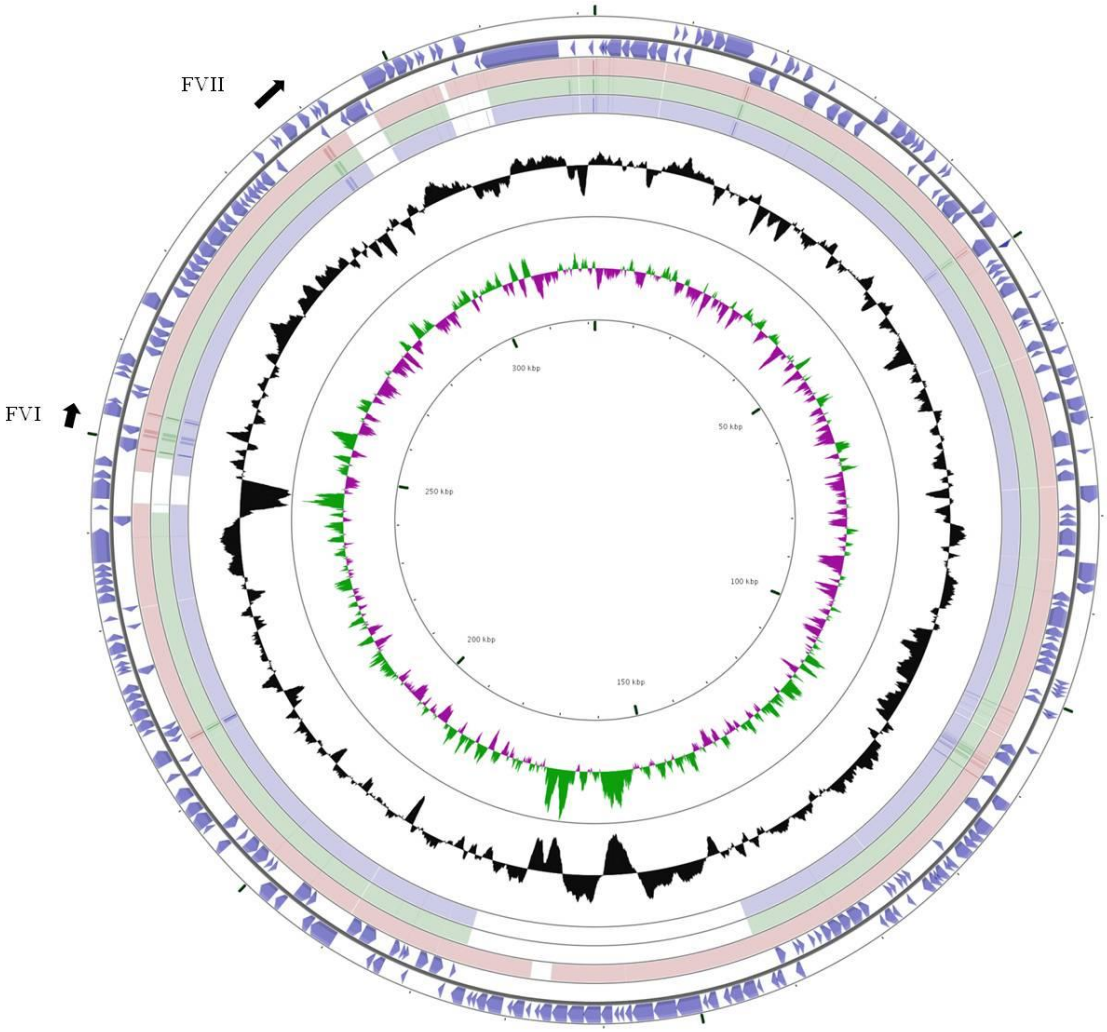

Figure 2.2: Genome comparison diagram of the LPP-1 plasmids of *P. ananatis*. As with the previous diagram, *P. ananatis* AJ13355 is presented by the outer two rings, LMG20103 in red, PA13 in green LMG5342 in blue. Two additional flagellar loci are located on the plasmid, namely FVI and FVII. These loci encode orthologous copies of the flagellin FliC.

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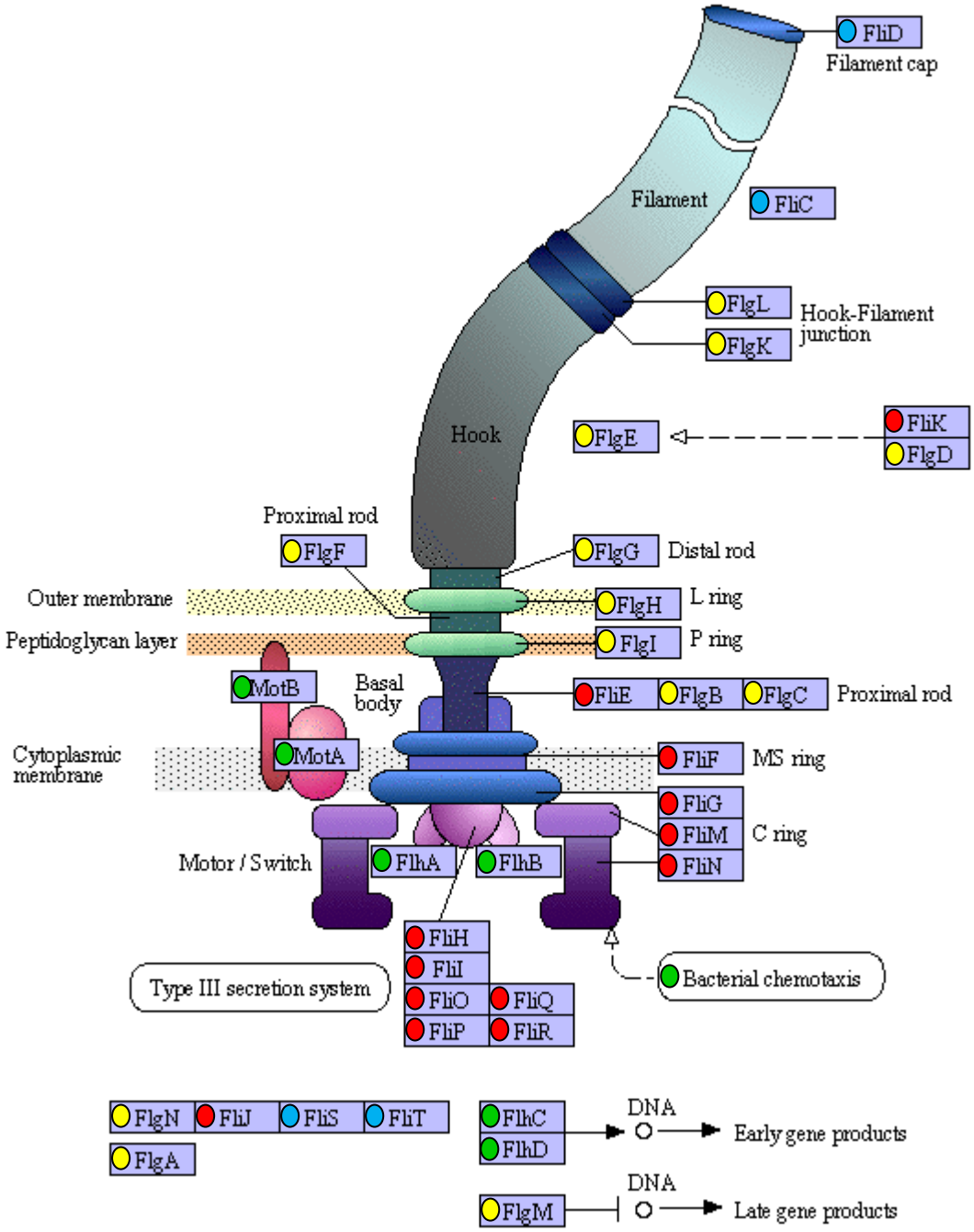

Figure 2.3: The flagellar biosynthetic loci of *P. ananatis.* Proteins indicated in yellow are found in locus FI and predominantly include the structural genes of the basal body and hook. The green proteins are located in FII and include the chemotaxis proteins, the motor proteins MotAB and the master regulator of the flagellar genes. All the proteins found in locus FIII are coloured blue. Proteins found in this locus include the flagellin protein, FliC, and the capping protein, FliD. The remainder of the motor and basal body structural proteins are located in FIV and are indicated in red (http://www.genome.jp/dbget-bin/www_bget?ko02040).

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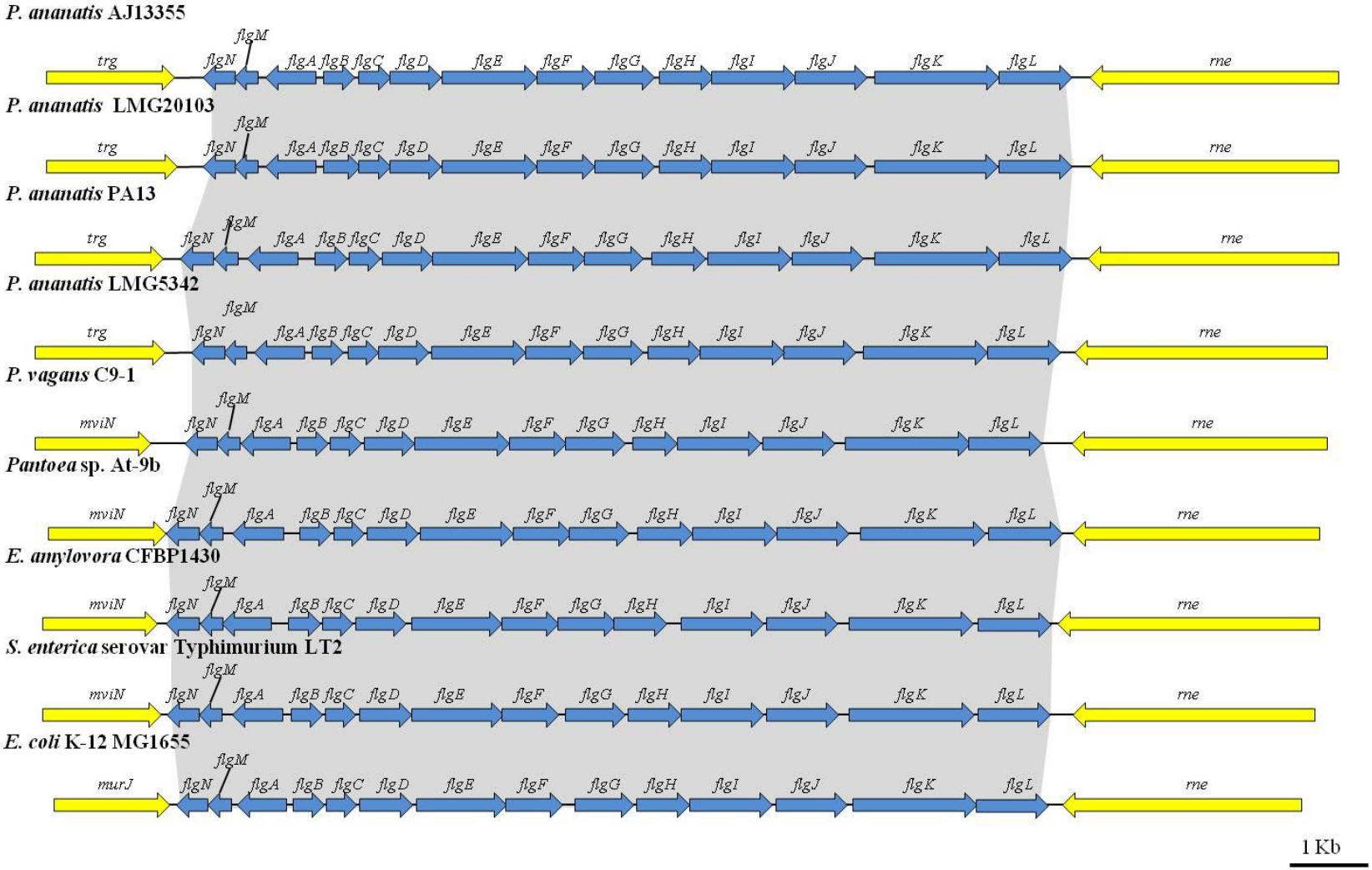

Figure 2.4: Flagella locus FI. As FI encodes many of the structural and assembly genes required for fully-functional flagella, the locus is conserved across all four sequenced *P. ananatis* strains, as well as the comparator strains.

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Figure 2.5: Flagella locus FII. Locus FII is conserved across the *P. ananatis* and *P. vagans* strains but differences in the other comparator strains were observed among the MCPs. Many of the motor and chemotaxis proteins are encoded in FII.

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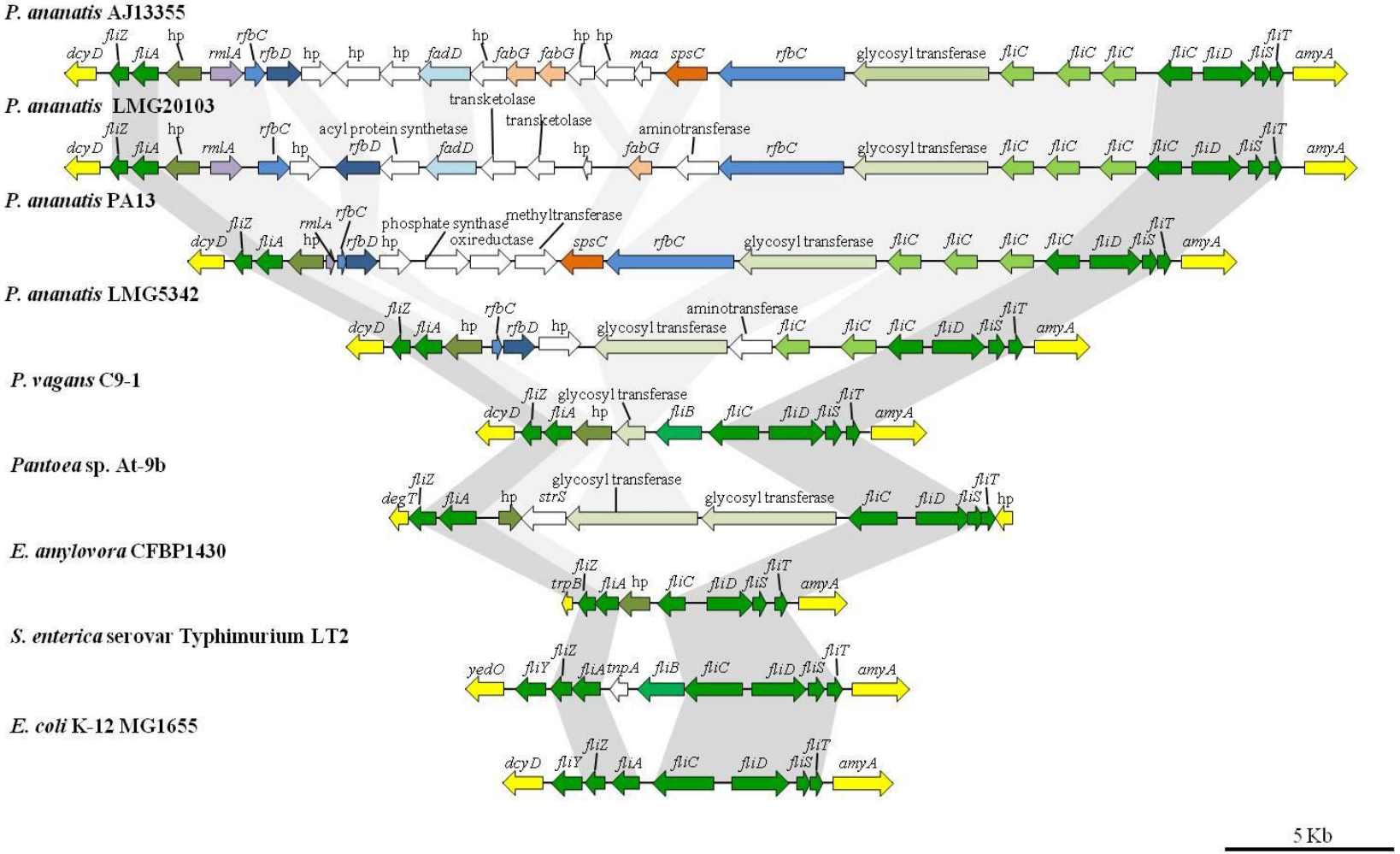

Figure 2.6: Flagella locus FIII. FliC1-4 are encoded in FIII thus this locus plays a role in flagellar phase variation in *P. ananatis*. The comparator strains each have only one copy of the *fliC* gene. FIII is the most variable amongst the flagellar loci as it encodes a non-conserved glycosylation island. Genes found in this locus include assembly and biosynthesis genes. hp = hypothetical protein.

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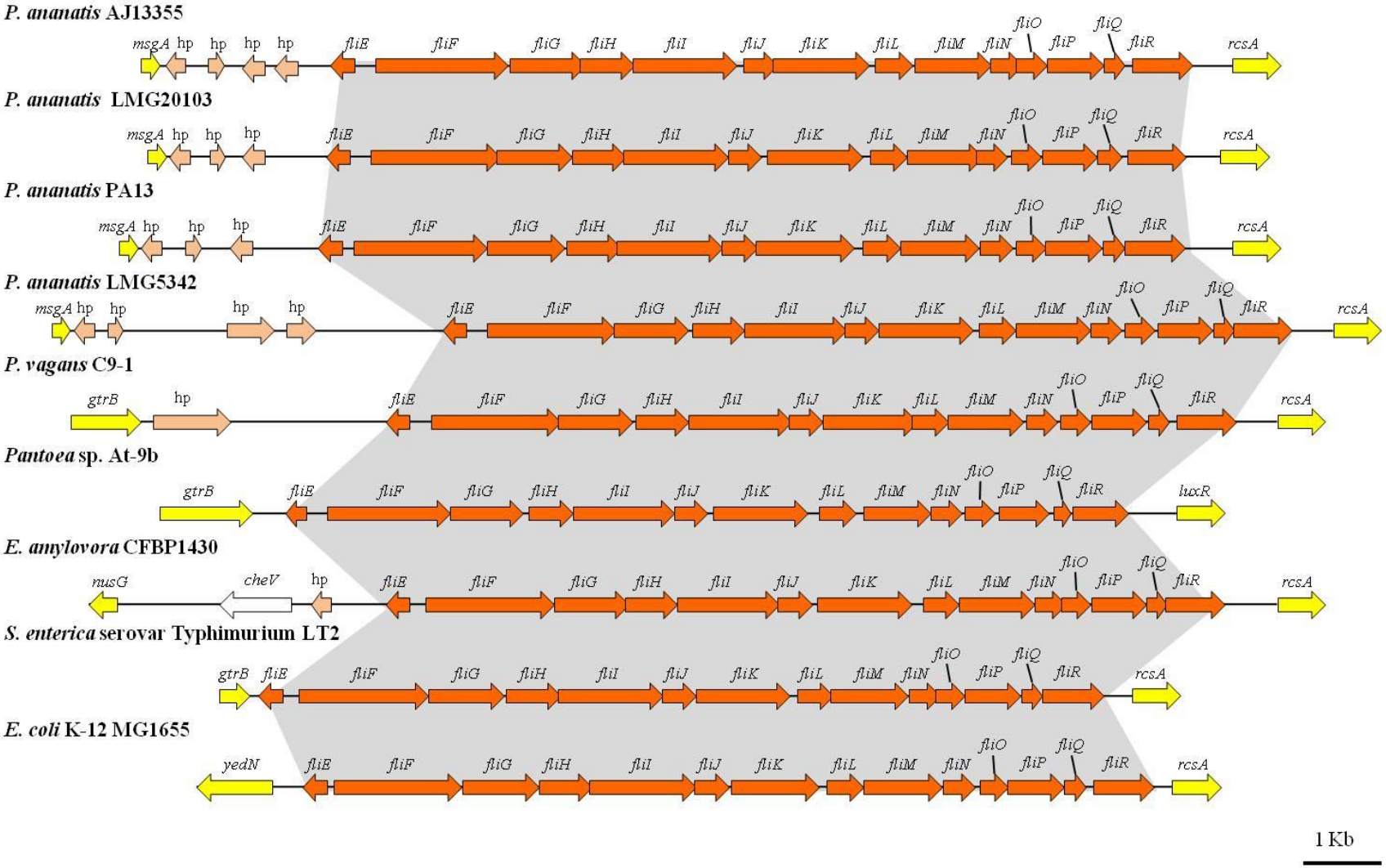

Figure 2.7: Flagella locus FIV. Locus FIV is conserved in all the compared strains. hp = hypothetical protein.

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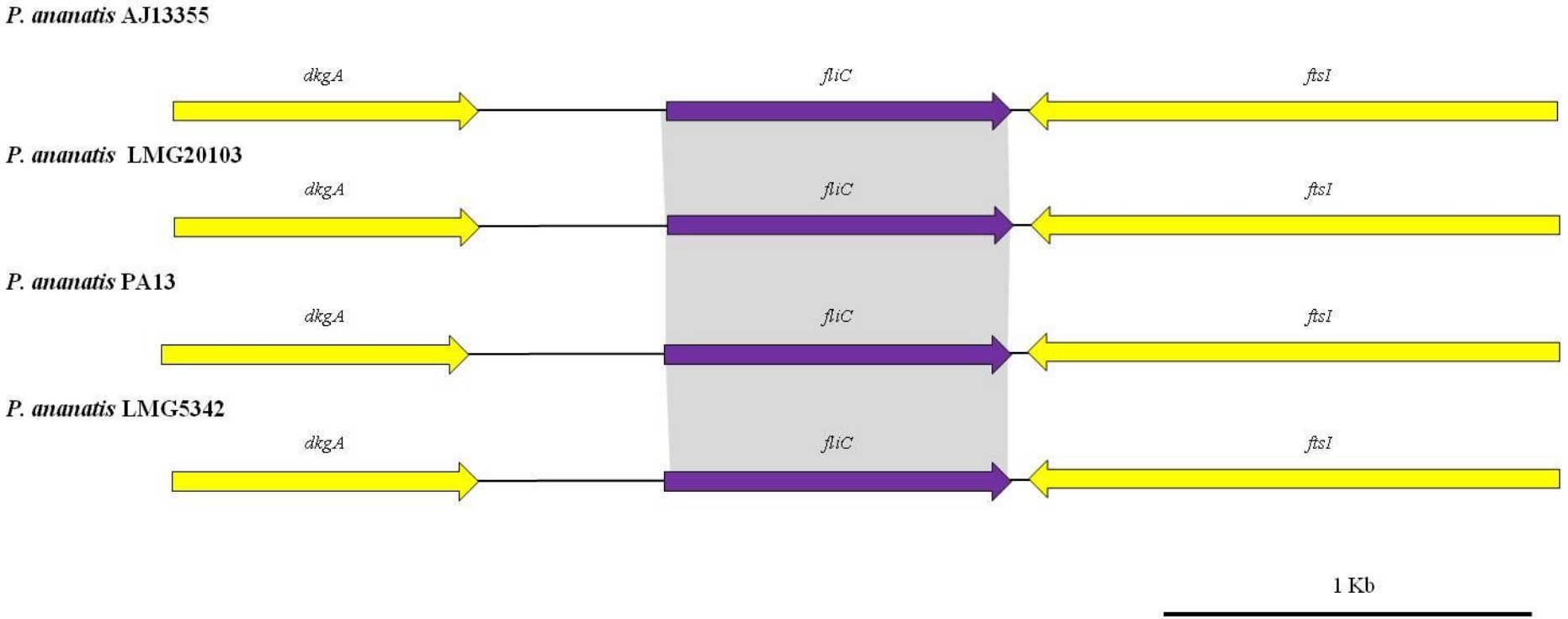

Figure 2.8: Flagella locus FV. All the *P. ananatis* strains included in this study carry a *fliC* gene at locus FV. The comparator strains each have one copy of the *fliC* gene and are thus missing FV.

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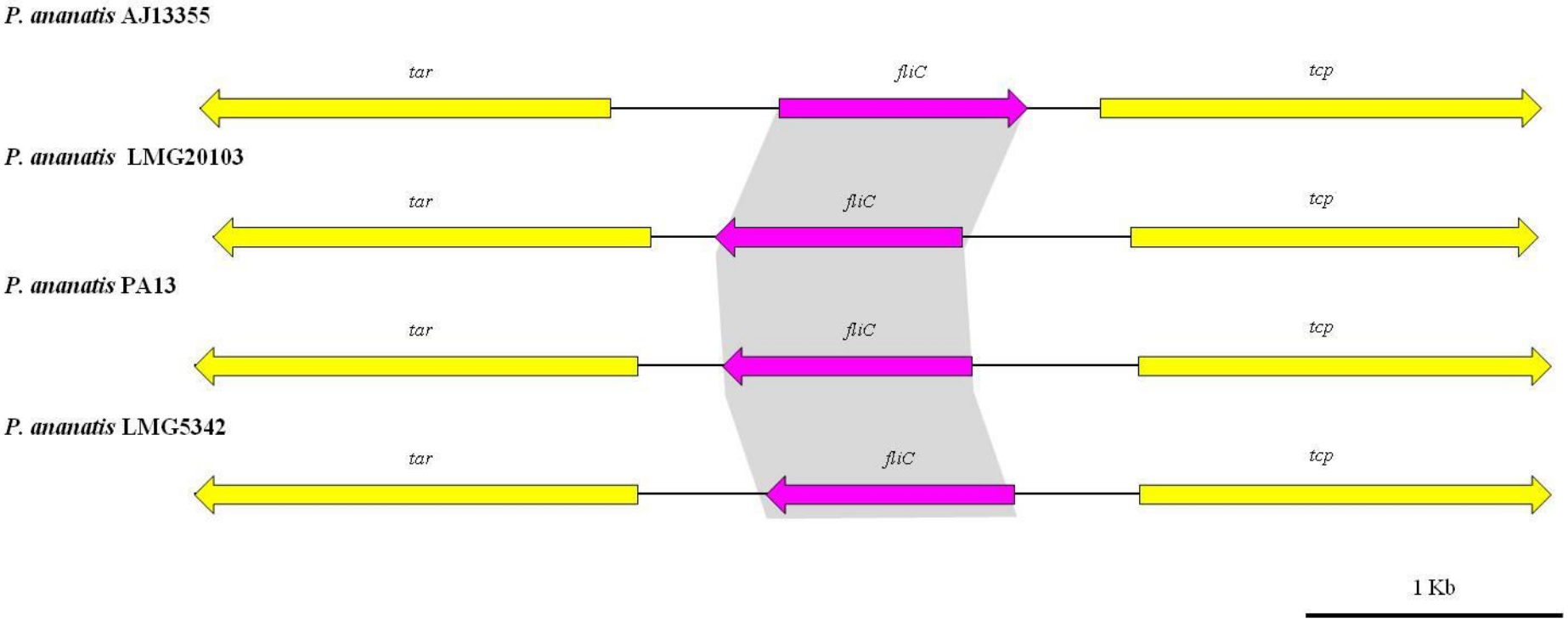

Figure 2.9: Flagella locus FVI. Each *P. ananatis* strains carries a copy of the *fliC* gene on its plasmid at locus FVI.

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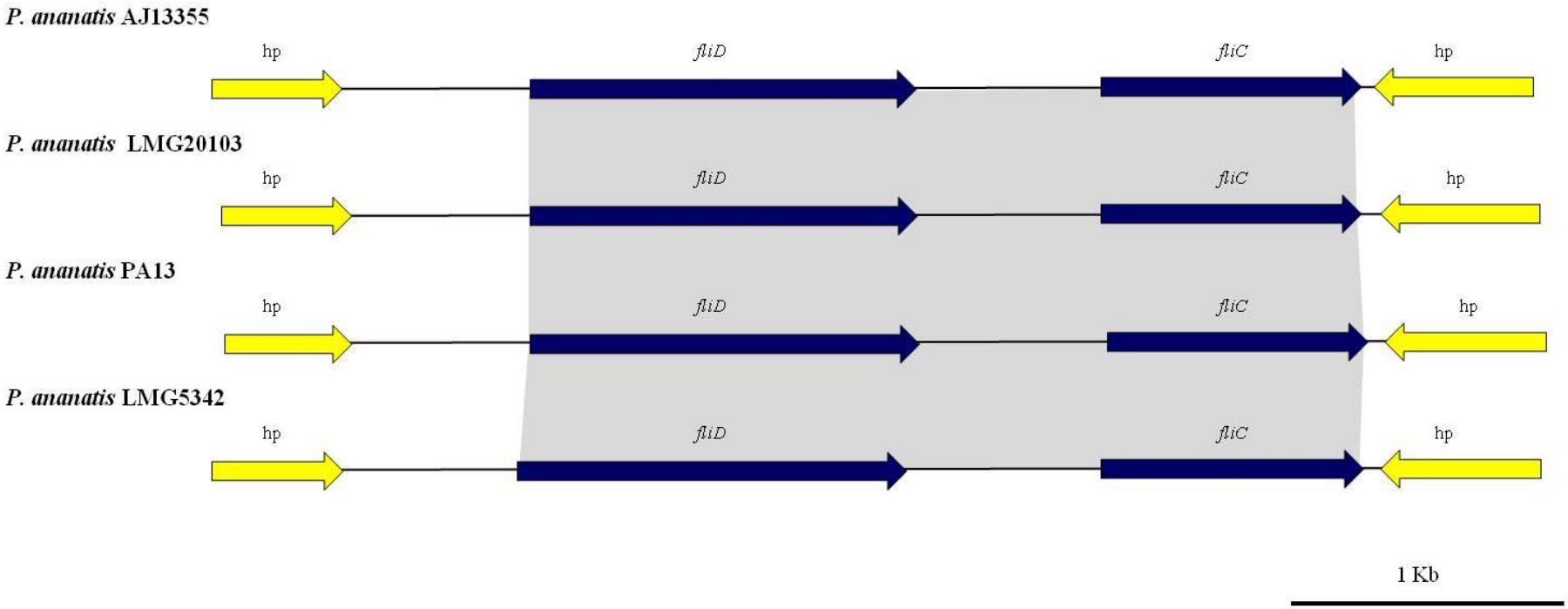

Figure 2.10: Flagella locus FVII. All four *P. ananatis* strains included in this study have an additional locus on their plasmid that codes for a copy of the capping protein and the final copy of flagellin. hp = hypothetical protein.

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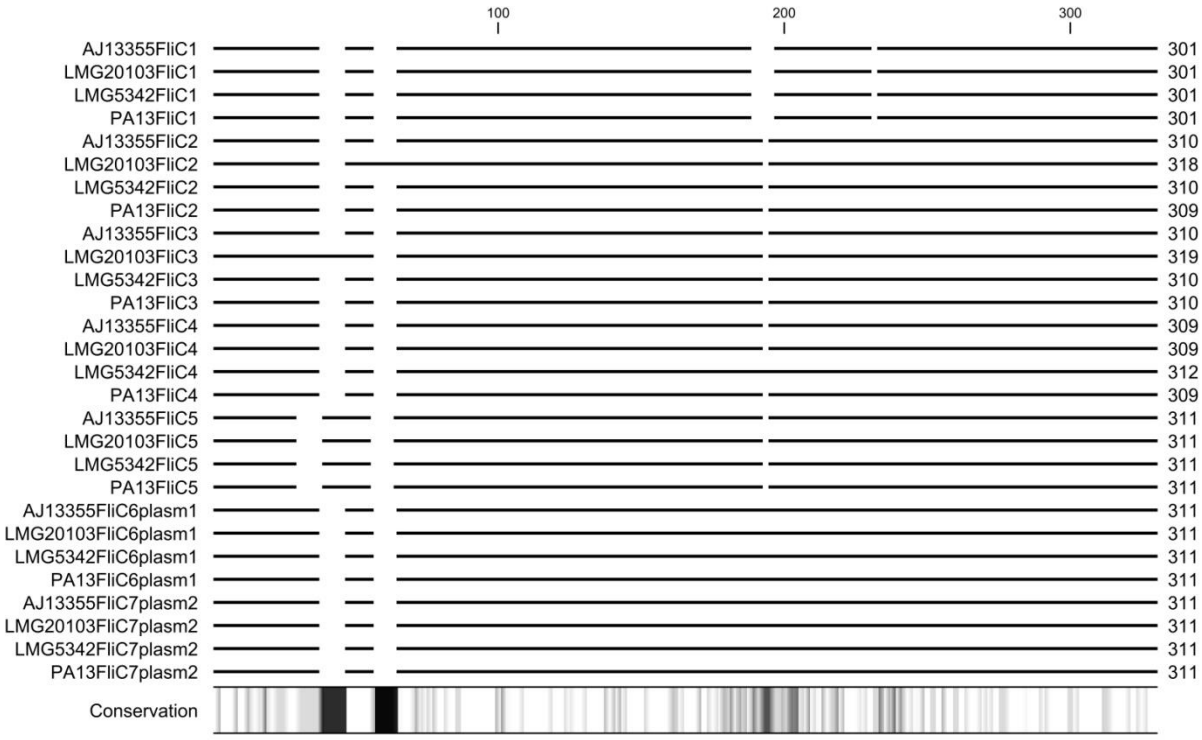

Figure 2.11: FliC variability in *P. ananatis*. Variability of the FliC proteins is predominantly found between amino acids 160 and 240. This correlates with the surface exposed portion of FliC and may be indicative of phase variation in *P. ananatis*. The FliC protein is, on average, 300 amino acids long.

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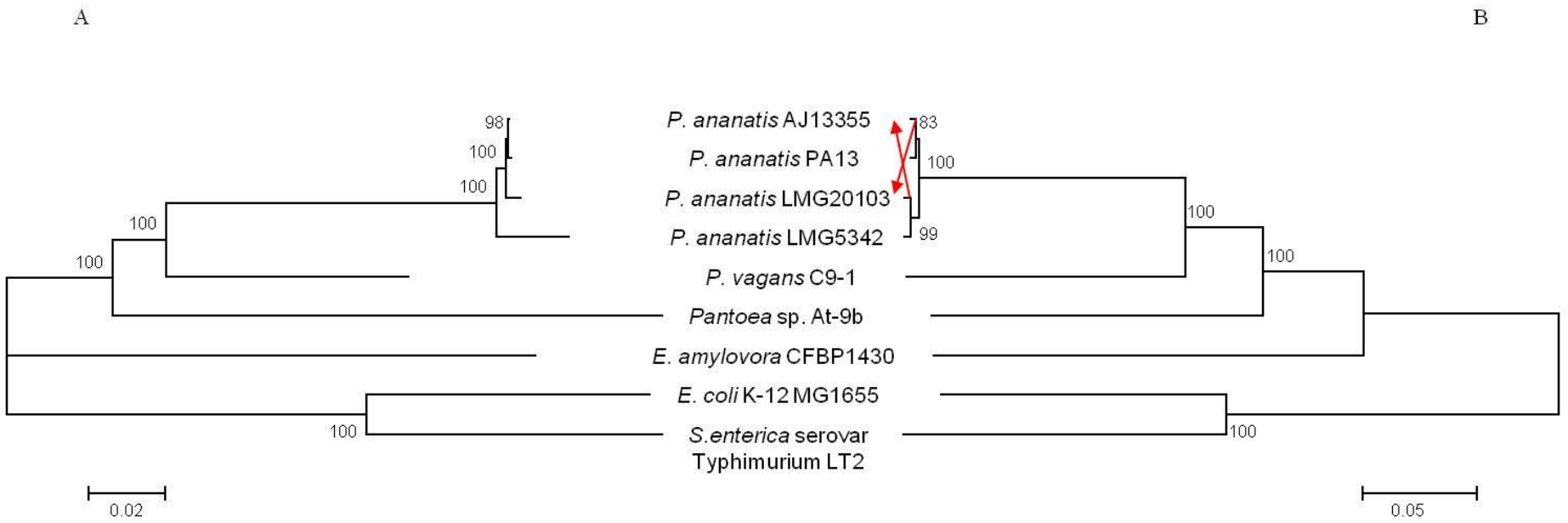

Figure 2.12: Neighbour-joining tree constructed on the basis of alignments of the concatenated amino acid sequences of the 47 core flagellar biosynthetic proteins (A) and the concatenated amino acid sequences of the four house-keeping markers AtpD, GyrB, InfB and RpoB (B). Differences in the clustering of the *P. ananatis* strains are indicated in red. The evolutionary distances indicated on the trees are in the units of the number of amino acid substitutions per site.

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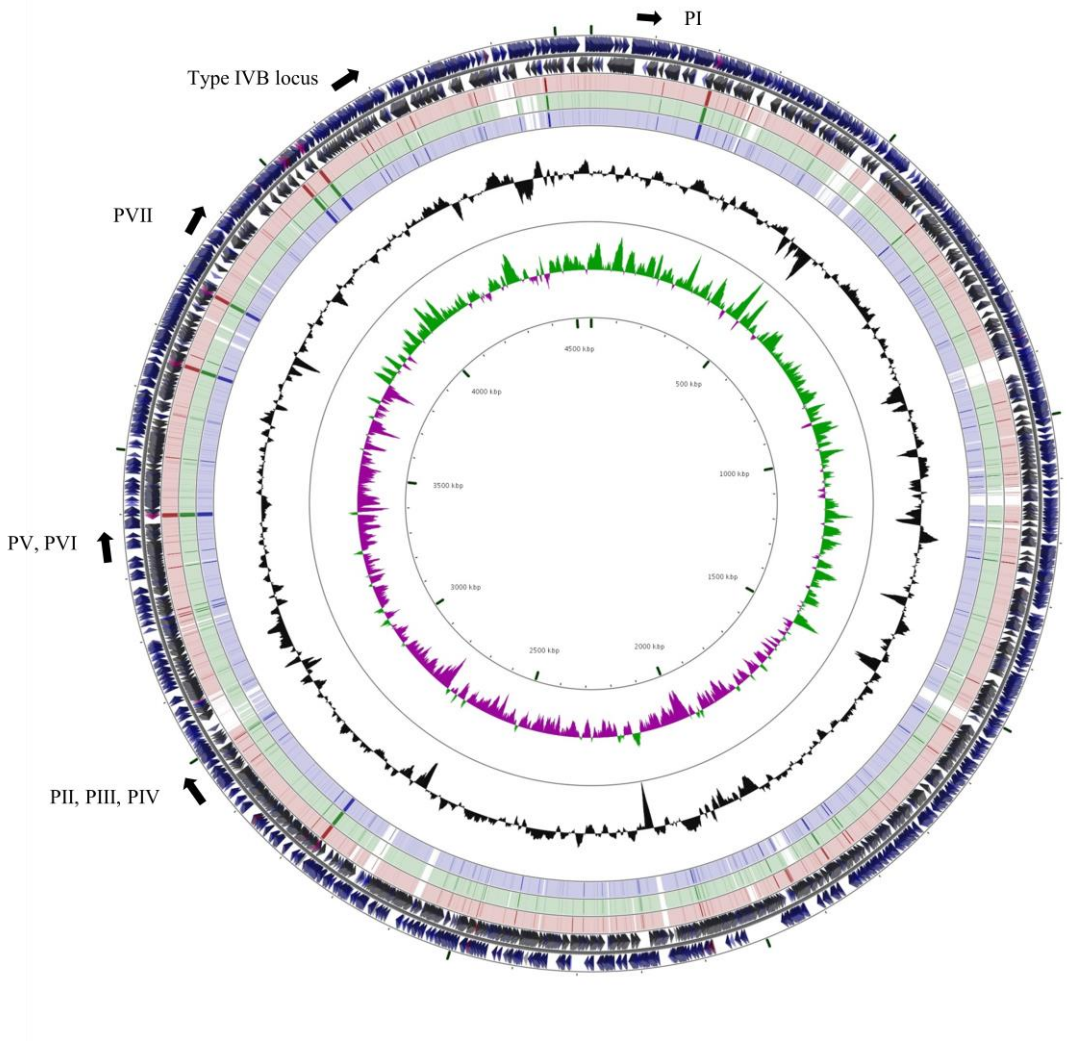

Figure 2.13: Genome comparison diagram of the chromosomes in *P. ananatis*. The type IV pilin genes are clustered into eight loci on the genome, indicated on the map as PI – PVII and the type IVB locus. The two outer rings depict the CDSs of *P. ananatis* AJ13355 in both a forward and reverse orientation whereas *P. ananatis* LMG20103 is in red, *P. ananatis* PA13 is in green and *P. ananatis* LMG5342 in blue.

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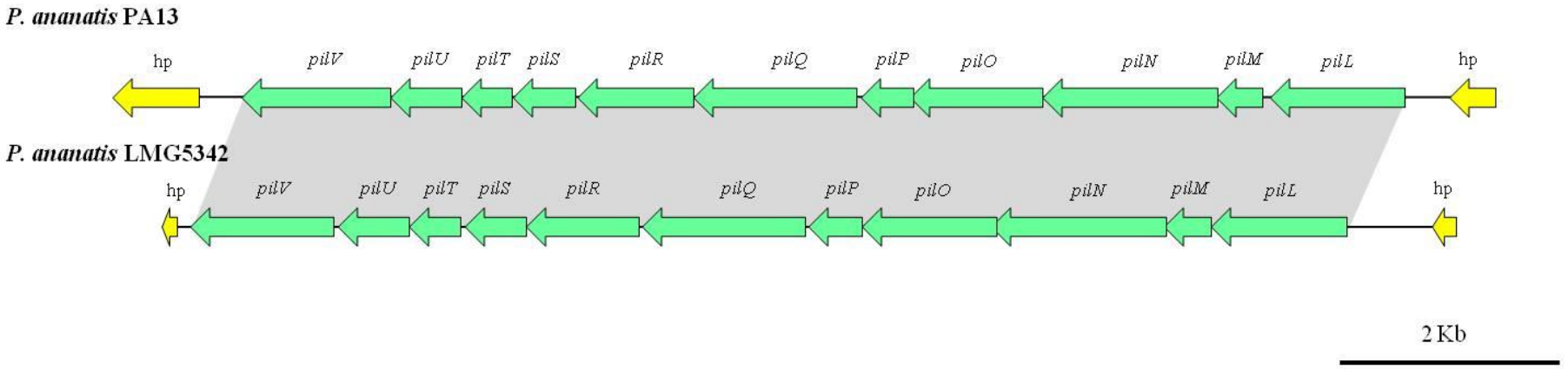

Figure 2.14: Type IVB pili locus. This locus encodes type IVB pili and is only present in *P. ananatis* PA13 and *P. ananatis* LMG5342. It is located on the ICE*Pan* and aids conjugation and the transfer of genetic material between cells. hp = hypothetical protein.

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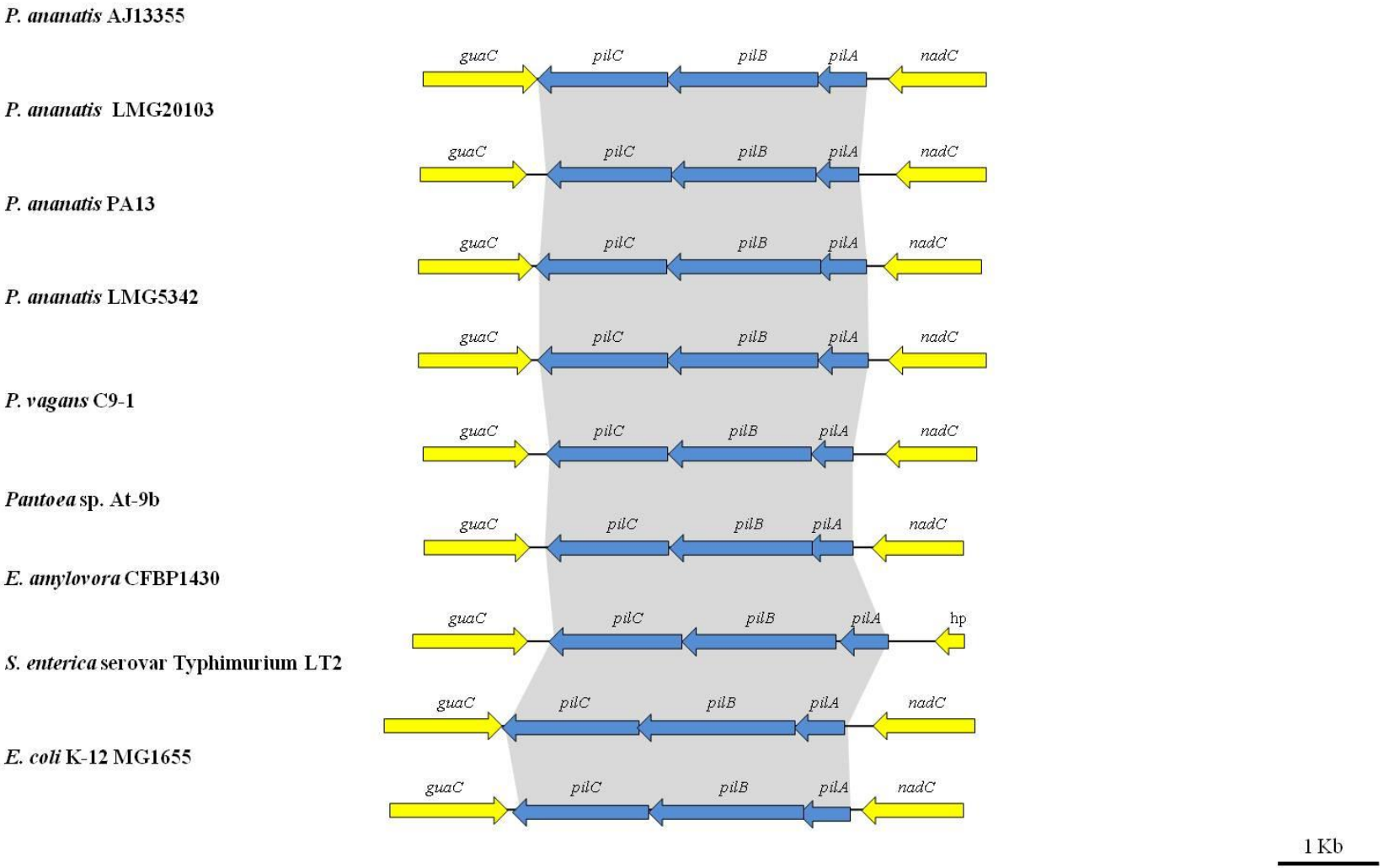

Figure 2.15: Type IV pili locus PI. Locus PI, which encodes the major pilin subunit, is highly conserved among all the compared strains. hp = hypothetical protein.

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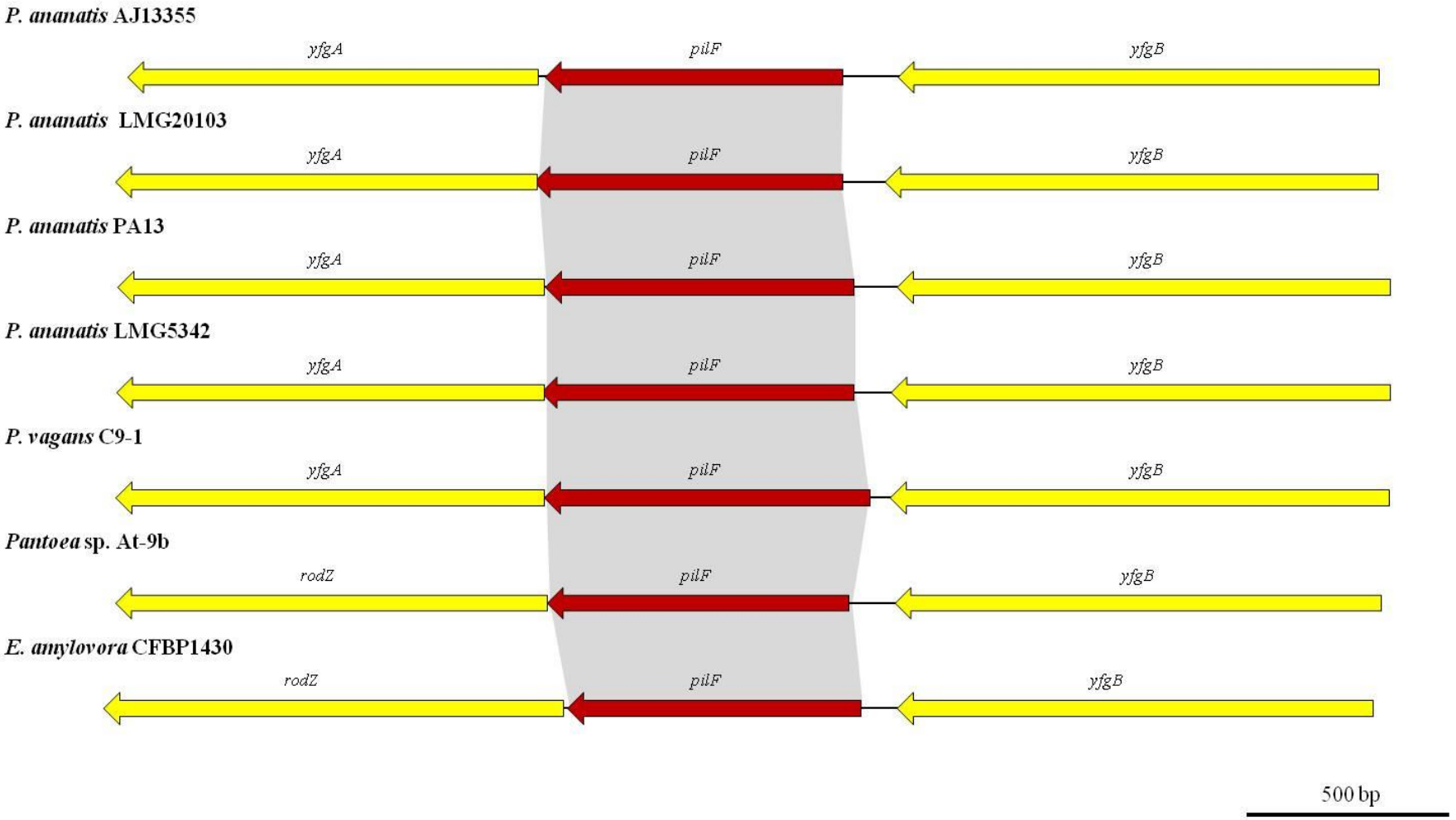

Figure 2.16: Type IV pili locus PII. PilF plays a role in the assembly of type IV pili and is thus a conserved protein, however, it is absent from *S. enterica* serovar Typhimurium LT2 and *E. coli* K-12 MG1655.

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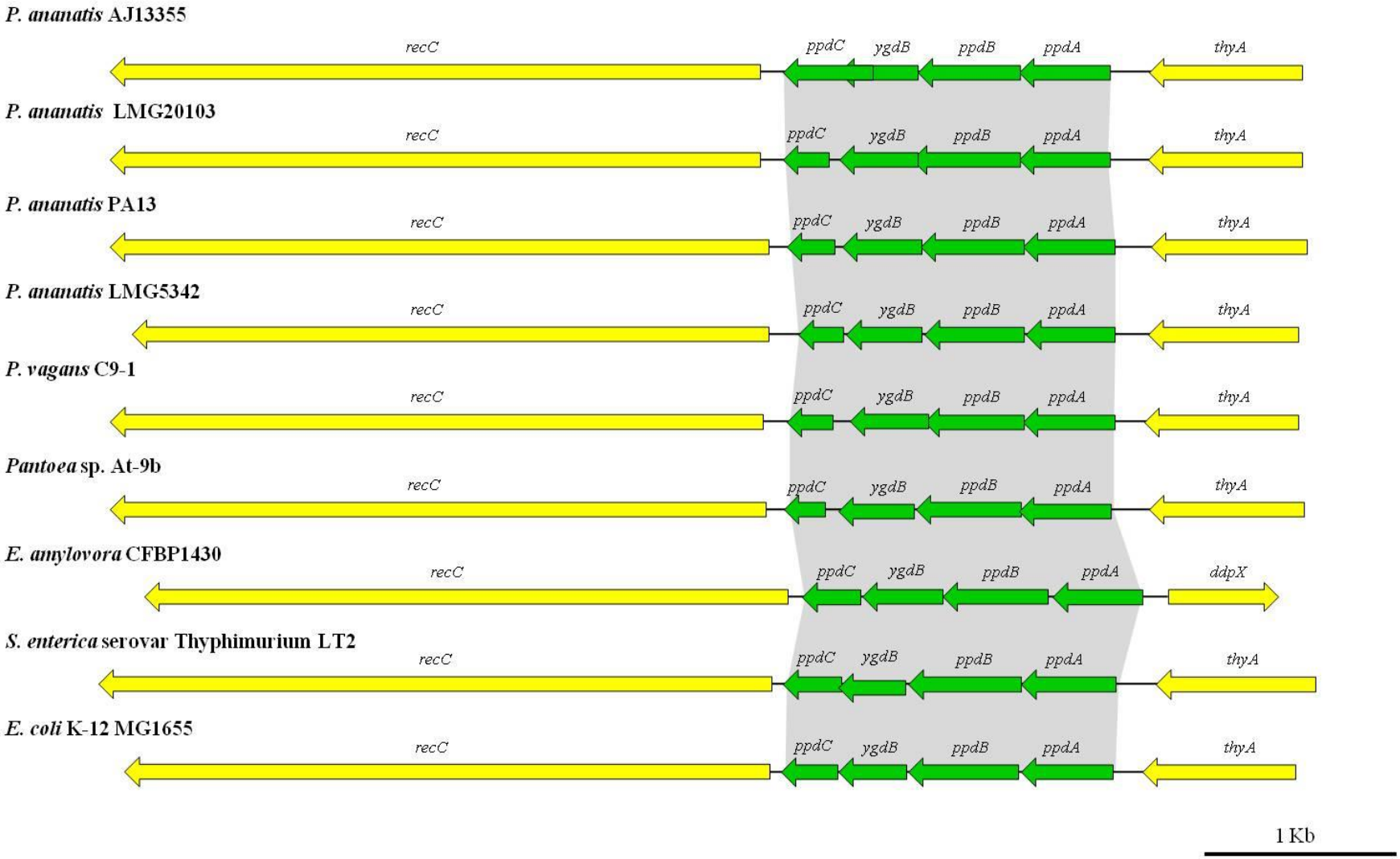

Figure 2.17: Type IV pili locus PIII. The prepilin peptidases cleave the leader sequence from the major pilin subunit.

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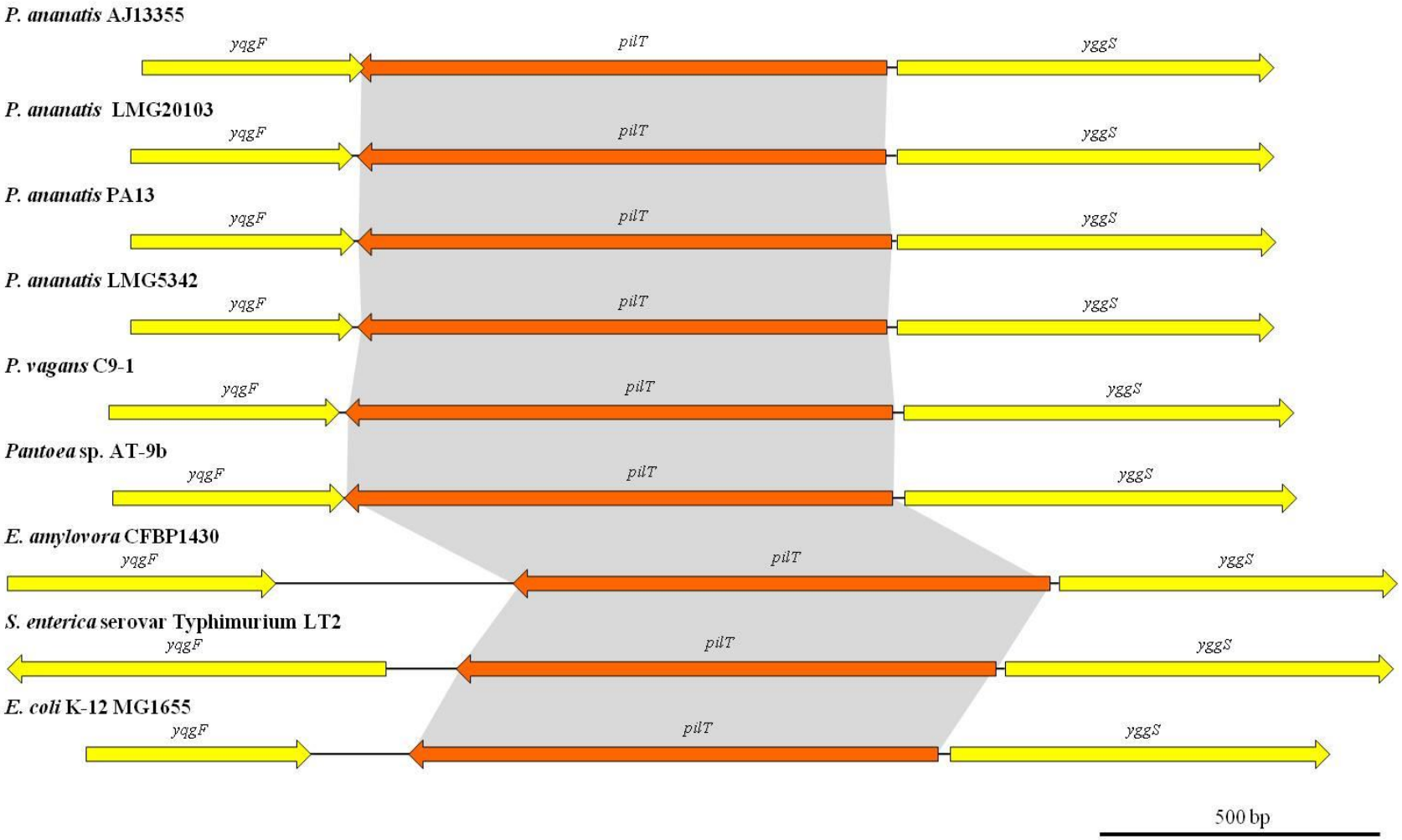

Figure 2.18: Type IV pili locus PIV. PilT is an ATPase that generates the energy needed for type IV pili to retract during twitching motility and is thus well conserved.

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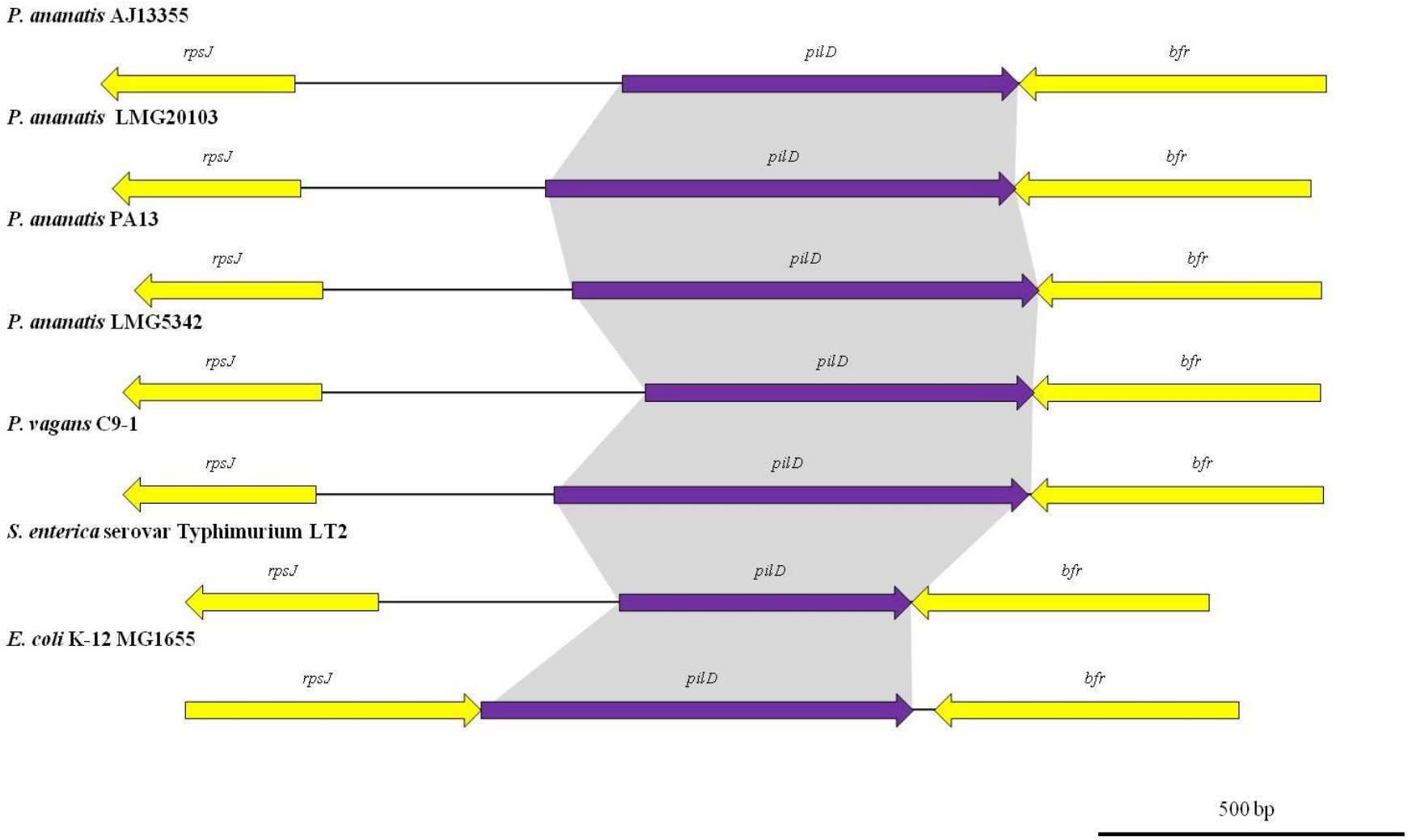

Figure 2.19: Type IV pili locus PV. PilD is a leader peptide processing enzyme and plays a role in type IV pili biogenesis.

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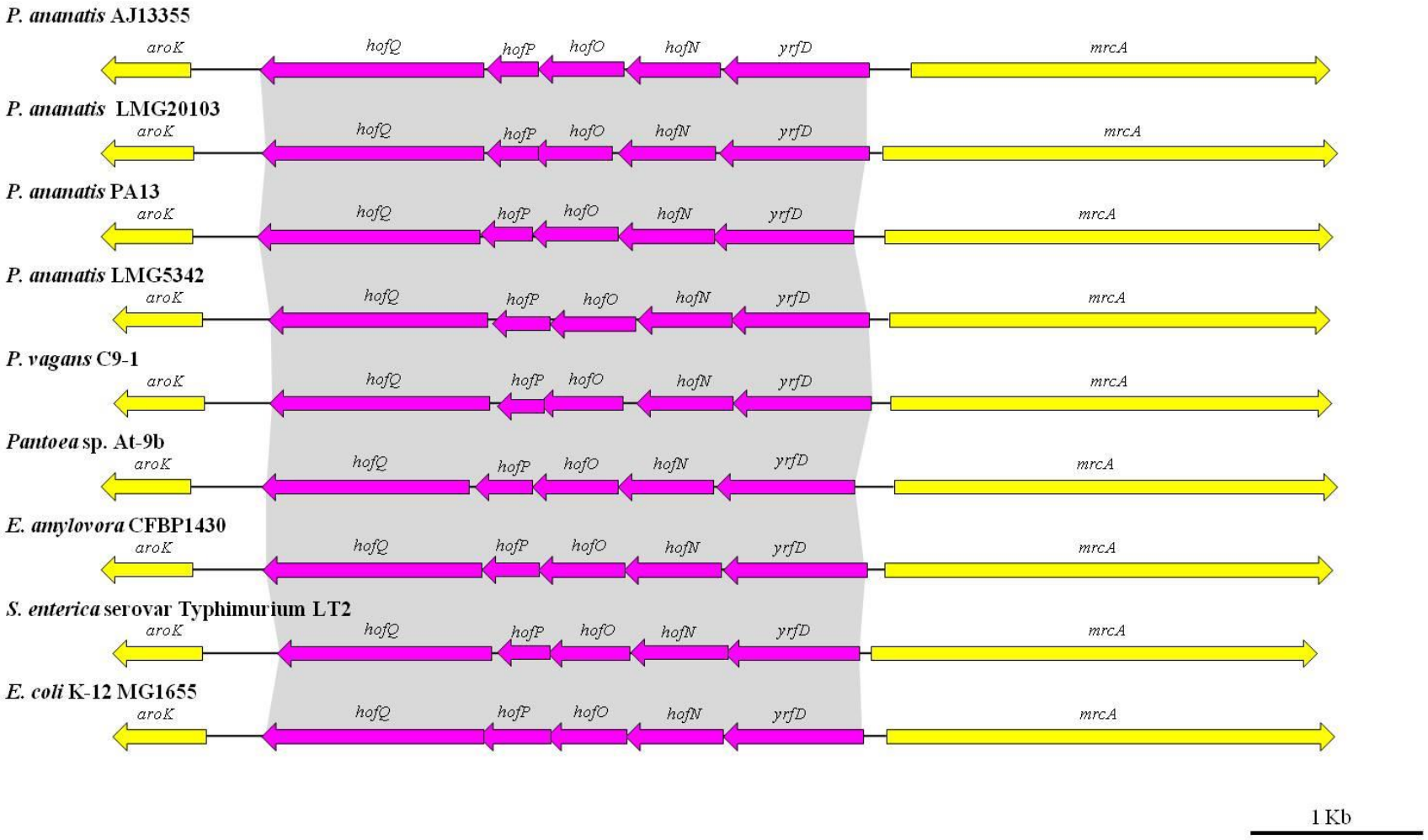

Figure 2.20: Type IV pili locus PVI. Locus PVI plays a role in assembly as HofQ is a secretin through which the pilin subunits are transported through the outer membrane. HofN aids in secretion. hp = hypothetical protein.

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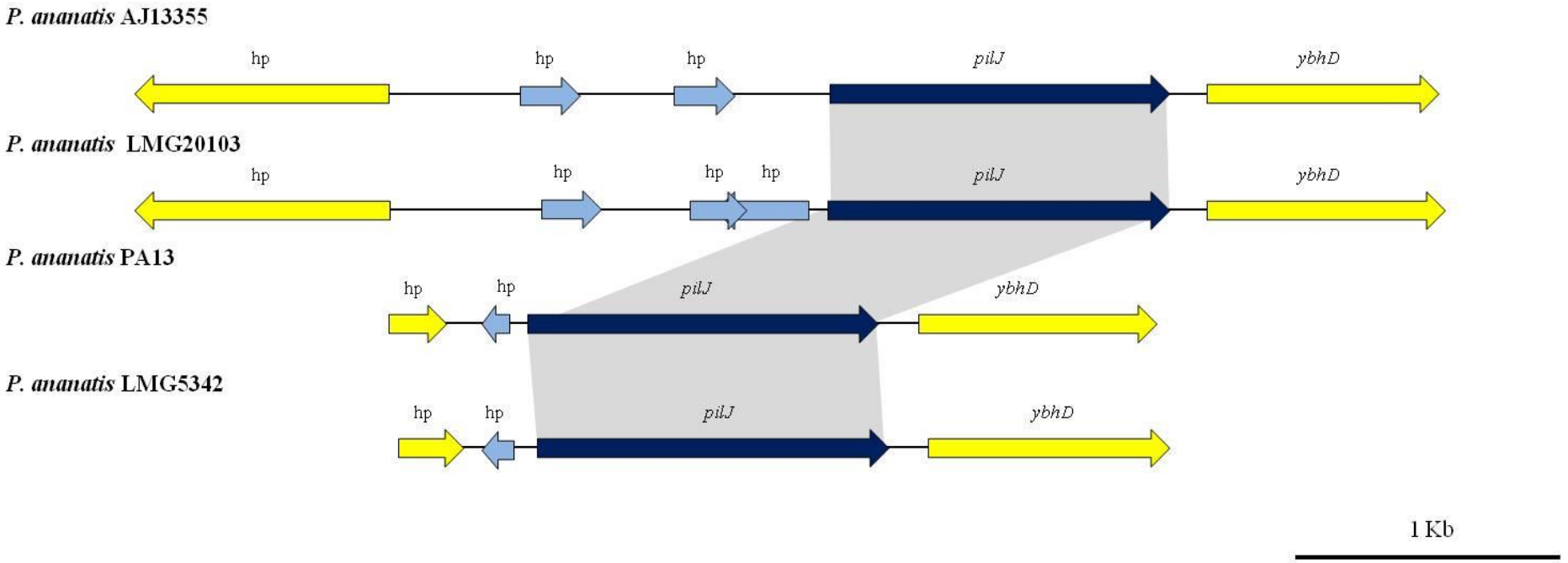

Figure 2.21: Type IV pili locus PVII. PilJ plays a role in pilus biogenesis and assembly and is only found in the *P. ananatis* strains. hp = hypothetical protein.

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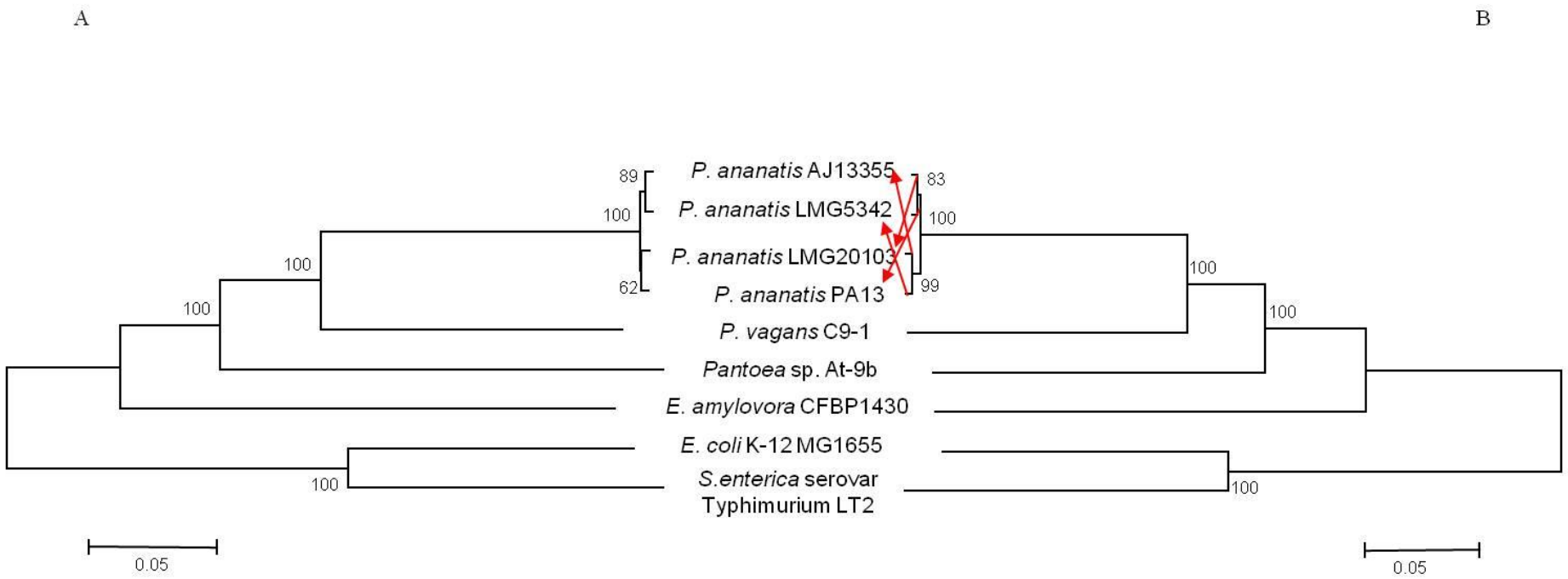

Figure 2.22: Neighbour-joining tree constructed on the basis of alignments of the concatenated amino acid sequences of the 13 core type IV pili biosynthetic proteins (A) and the concatenated amino acid sequences of the four house-keeping markers AtpD, GyrB, InfB and RpoB (B). Differences in the clustering of the *P. ananatis* strains are indicated in red. The evolutionary distances indicated on the trees are in the units of the number of amino acid substitutions per site.

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TABLES

Table 2.1: Genome properties of the *P. ananatis* strains used in the comparative analyses

Strain	Source	NCBI Acc Number	Chromosome			Plasmid		
			Size (Mb)	CDS	G+C (%)	Size (Kb)	CDS	G+C (%)
AJ13355	Soil	NC_017531	4.56	4019	53.8	322	272	53.4
LMG20103	*Eucalyptus*	CP001875	4.39	3955	53.7	317	270	52.1
LMG5342	Human	HE617160	4.61	4098	53.5	303	255	51.5
PA13	Rice	CP003085	4.59	4156	53.7	281	238	52.3

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Table 2.2: Percentage average amino acid identities of the flagellar proteins in *P. ananatis* and enterobacterial comparator strains.

	***P. ananatis* AJ13355**	***P. ananatis* LMG20103**	***P. ananatis* PA13**	***P. ananatis* LMG5342**	***P. vagans* C9-1**	***Pantoea* sp. At-9b**	***E. amylovora* CFBP1430**	***S. enterica* serovar Typhimurium LT2**	***E. coli* K-12 MG1655**
***P. ananatis* AJ13355**	100	99.33	99.81	99.78	84.22	81.56	73.39	68.48	68.10
***P. ananatis* LMG20103**	99.33	100	99.19	99.23	83.45	80.92	73.74	69.12	67.73
***P. ananatis* PA13**	99.81	99.19	100	99.65	84.66	81.85	74.13	68.44	67.94
***P. ananatis* LMG5342**	99.78	99.23	99.65	100	84.20	81.21	73.94	69.31	68.21
***P. vagans* C9-1**	84.22	83.45	84.66	84.20	100	84.60	73.34	69.24	70.09
***Pantoea* sp. At-9b**	81.56	80.92	81.85	81.21	84.60	100	74.77	69.28	70.40
***E. amylovora* CFBP1430**	73.39	73.74	74.13	73.94	73.34	74.77	100	69.64	70.26
***S. enterica* serovar Typhimurium LT2**	68.48	69.12	68.44	69.31	69.24	69.28	69.64	100	83.28
***E. coli* K-12 MG1655**	68.10	67.73	67.94	68.21	70.09	70.40	70.26	83.28	100

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Table 2.3: Pili present in *P. ananatis*

	AJ13355	LMG5342	LMG20103	PA13
Chaperone-usher pathway				
Type I pili				
Stb pili	+	+	+	+
Csu pili	+	+	+	+
MRF	+	+	+	+
P pili	-	+	-	-
Unclassified type I pilus	+	-	-	-
Type III pili				
Mrk pili	+	+	+	+
General secretion pathway				
Type IV pili				
Type IVA pili	+	+	+	+
Type IVB pili	-	+	-	+

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Table 2.4: Percentage average amino acid identities of the pilin proteins in *P. ananatis* and enterobacterial comparator strains

	***P. ananatis* AJ13355**	***P. ananatis* LMG20103**	***P. ananatis* PA13**	***P. ananatis* LMG5342**	***P. vagans* C9-1**	***Pantoea* sp. At-9b**	***E. amylovora* CFBP1430**	***S. enterica* serovar Typhimurium LT2**	***E. coli* K-12 MG1655**
***P. ananatis* AJ13355**	100	95.10	94.92	95.13	70.79	66.26	56.94	47.85	51.60
***P. ananatis* LMG20103**	95.10	100	99.40	99.12	71.03	66.65	59.06	47.94	51.44
***P. ananatis* PA13**	94.92	99.40	100	99.35	71.07	66.49	59.16	48.04	51.48
***P. ananatis* LMG5342**	95.13	99.12	99.35	100	70.78	66.42	58.87	48.11	51.54
***P. vagans* C9-1**	70.79	71.03	71.07	70.78	100	70.23	59.00	49.32	51.25
***Pantoea* sp. At-9b**	66.26	66.65	66.49	66.42	70.23	100	61.88	51.4	52.00
***E. amylovora* CFBP1430**	56.94	59.06	59.16	58.57	59.00	61.88	100	49.29	50.99
***S. enterica* serovar Typhimurium LT2**	47.85	47.94	48.04	48.11	49.32	51.40	49.29	100	75.34
***E. coli* K-12 MG1655**	51.60	51.44	51.48	51.54	51.25	52.00	50.99	75.34	100

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CHAPTER 3

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CHAPTER THREE

CONSTRUCTING FLAGELLA (*flgK*$^-$ and *motA*$^-$) AND TYPE IV PILI (*pilA*$^-$ and *pilT*$^-$) MUTANTS AND THEIR RESPECTIVE COMPLEMENTS IN *PANTOEA ANANATIS* LMG20103

3.1 INTRODUCTION

Pantoea ananatis is a Gram-negative phytopathogen belonging to the Enterobacteriaceae. It has a very broad host range and is capable of infecting a variety of crops such as *Eucalyptus* (Coutinho *et al*., 2002), maize (Gosczcynska *et al*., 2007; Lana *et al*., 2012), rice (Watanabe *et al*., 1996) and onion (Gitaitis *et al*., 2002; Gosczcnska *et al*., 2006). Most strains of *P. ananatis* are non-host specific and are capable of infecting a number of different hosts (Gosczcynska *et al*., 2007). *P. ananatis* is both an epiphyte and endophyte and has also been isolated from a wide variety of environments that range from soil to aviation fuel tanks (Lai and Hsu, 1974; Rauch *et al*., 2006) and insects such as ticks and tobacco thrips (Murrell *et al*., 2003). It is a necrogenic plant pathogen that causes plant cell death, although many of its hosts do not display any symptoms. The pathogenicity of *P. ananatis* is poorly understood, although comparisons to well-characterised plant pathogens such as *Erwinia amylovora* and *Pectobacterium carotovorum* have shown orthologous pathogenicity determinants including flagella, fimbriae and non-fimbrial adhesins, ice nucleation proteins and potential cell wall degrading enzymes (de Maayer *et al*., 2014). *P. ananatis* also has a type VI secretion system which aids in pathogenicity and competition between *P. ananatis* and other bacterial species (de Maayer *et al*., 2011; Shyntum *et al*., 2014).

The ability of pathogenic bacteria to rapidly spread between and within host plants is largely dependent on its motility. Motility is a key factor in establishing an infection in the host plant once the bacteria have attached to the host surface (Josenhans and Suerbaum, 2002; Soutourina and Bertin, 2003; Meng *et al*., 2011). The fastest form of motility amongst plant pathogens is swimming motility, which is enabled by the flagellum (Sun *et al*., 2000). Flagella are important not only in swimming motility, but have also been shown to play a role in attachment, biofilm formation (O'Toole and Kolter, 1998; Kang *et al*., 2002; Meng *et al*.,

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2011; Haiko *et al*., 2013) and virulence (Shen and Ronald, 2002; Haiko *et al*., 2013). Virulence-associated proteins are also known to be secreted through the flagella filament (Kirov, 2003, Fernando *et al*., 2007). It is therefore hypothesised that flagella in *P. ananatis* play an important role in infection and colonisation of host plants.

Twitching motility allows bacteria to rapidly colonise a surface area (Liu *et al*., 2001) through the retraction of type IV pili (Li *et al*., 2007). It has been found that twitching motility is a quantitative trait that increases as the cell density increases (Meng *et al*., 2011). As with flagella, type IV pili are widespread and are found in many plant (Pratt and Kolter, 1998; Sun *et a*l., 2000; Liu *et al*., 2001; Bahar *et al*., 2009), and human pathogens (Alm and Mattick, 1997; Cleary *et al*., 2004; Fraser and Hughes, 1999). In *Pseudomonas aeruginosa* (Shen *et al*., 2012) and *Ralstonia solanacearum* (Liu *et al*., 2001) it plays an important role in the spread of the pathogen within the host plant. Type IV pili also play a role in biofilm formation (Liu *et al*., 2001; Kang *et al*., 2002), virulence (Kang *et al*., 2002; Bahar *et al*., 2009; Burdman *et al*., 2011), cell-cell aggregation (Liu *et al*., 2001; Kang *et al*., 2002), attachment and bacteriophage adsorption (Soto and Hultgren, 1999).

To investigate the role of motility in the pathogenicity and fitness of *P. ananatis*, several genes will be disrupted to ascertain the effect of these disruptions on the cell's phenotype. There are several methods available to elucidate the effect that certain genes have on the phenotype of a cell, however, many of them are laborious and time-consuming, as well as being relatively vulnerable to human error. An example is random transposon mutagenesis which has been used extensively to define the essential and pathogenicity-related genes in the genomes of both prokaryotes and eukaryotes (Hamer *et al*., 2001). This method, however, represents a hit and miss approach, where laborious phenotypic screening assays of hundreds or thousands of mutants must be undertaken to find those strains carrying the desired mutation. Novel site-directed techniques which are rapid and easy to apply have recently been developed. One such technique, recombineering, or recombination-mediated genetic engineering, is an *in vivo* method of target mutagenesis and makes use of the bacteriophage λ homologous recombination system (Katshkina *et al*., 2009).

The aims of this chapter were to construct two flagella and two type IV pili mutants through genetic recombineering. The disruption of the flagellar genes *flgK* and *motA* rendered both strains unable to swim with the *flgK*$^{-}$ strain being aflagellate and the *motA*$^{-}$ strain having

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flagella that are unable to rotate, essentially paralysing the cells. The type IV pili genes that were disrupted in LMG20103 were *pilA* and *pilT*. The mutant strains were deficient in twitching motility. Specifically, the *pilA*$^{-}$ mutant was unable to generate type IV pili whereas a deletion of the *pilT* gene resulted in type IV pili remaining fully extended and unable to retract. Each mutant strain was complemented by restoring the functionality of the deleted gene in the respective mutated strain. The mutant and complemented strains will be used in further downstream tests and assays in comparison to the wild-type strain to elucidate the role of swimming and twitching motility in the plant-pathogenic lifestyle of *P. ananatis*.

3.2 MATERIALS AND METHODS

Unless stated otherwise, all the bacterial strains used in this study were cultured in LB broth (1% [w/v] tryptone, 0.5% [w/v] yeast extract, 0.5% [w/v] NaCl; pH 7.2) or on LB agar (LB broth amended with 1.2% [w/v] agar; pH 7.2) at 28°C. The media was supplemented with kanamycin (50 µg/ml), ampicillin (50 µg/ml), chloramphenicol (50 µg/ml) and gentamicin (50 µg/ml) as required. The strains and plasmids used in this study are listed in Table 3.1. The wild-type strain, *P. ananatis* LMG20103, was originally isolated from *Eucalyptus* in South Africa in 1998 (Coutinho *et al*., 2002).

3.2.1 Antibiotic sensitivity assays in *P. ananatis* LMG20103

Preliminary evidence suggested that *P. ananatis* is resistant to a wide range of antibiotics (De Baere *et al*., 2004) and thus its sensitivity and resistance to antibiotics was tested in order to identify a selective marker for constructing motility deletion mutants. *P. ananatis* has been treated successfully in humans using combinations of piperacillin-tazobactam, ofloxacin, cotrimoxazol, cefuroxime, furadantin and temocillin (De Baere *et al*., 2004). The antibiotic sensitivity of *P. ananatis* LMG20103 was subsequently tested as follows:

1. Stock solutions of gentamycin, tetracycline, ampicillin, kanamycin, streptomycin and neomycin were prepared according to the manufacturers' instructions (Sigma-Aldrich and Fluka) and diluted in sterile distilled water (dH_2O) to final concentrations as shown in Table 3.6. Filter paper discs were autoclaved and dried in an oven. The discs were soaked in the final concentration of each antibiotic and allowed to dry on a sterile surface in a laminar flow cabinet. LB agar was inoculated with a culture of *P.*

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ananatis LMG20103 by spreading 200 µl of the culture across the surface with a sterile spreader, and the antibiotic discs were placed on the surface of the agar using aseptic techniques. The plates were incubated overnight at 28°C after which the plates were examined for zones of inhibition.

2. Antibiograms are sterile paper rings that are impregnated with different antibiotics at regular intervals. *P. ananatis* LMG20103 was spread plated onto LB agar before a 25-TRA-1 Microring TRA1 (Abtek) and a Mastring-S M11 (Mast Diagnostics) antibiogram was aseptically placed on the surface of the agar. The antibiotics that were tested, as well as the concentration of each antibiotic, are shown in Table 3.7. The plates were incubated overnight at 28°C.

3. *P. ananatis* LMG20103 was spread plated onto LB agar that had been amended with either kanamycin or tetracycline, in concentrations of 25, 50 and 75 µg/ml, respectively. The plates were incubated overnight at 28°C and examined for bacterial growth.

3.2.2 Gene selection for motility deletion mutants

To differentiate between flagellar motility and flagellar attachment, two separate flagellar genes were selected to create swimming mutants. *flgK* encodes a hook associated protein that is essential for the assembly and functioning of the flagellum. Without it the flagellum cannot be properly assembled and mutants are incapable of swimming (Homma *et al*., 1990; O'Toole and Kolter, 1998). The *fliC* gene, coding for the major flagellin structural protein, was not selected for the functional flagellar mutations as *P. ananatis* LMG20103 has seven highly conserved copies of *fliC* on its genome (de Maayer *et al*., 2012) which is likely to result in complementation of the disrupted copy by a functional ortholog. The second target gene selected was a flagellar motor gene, *motA* (Josenhans and Suerbaum, 2002). MotA is involved in establishing the proton motive force that powers the flagellum (DeRosier, 1998; Madigan *et al*., 2003). Mutations in either *motA* or *motB* allow for complete flagellar synthesis, but the flagella are unable to rotate and are essentially paralysed (Pratt and Kolter, 1998; Ramos *et al*., 2004). A mutation in *motA* will therefore shed light on whether the flagella are important in attachment as well as motility. Both *flgK* and *motA* are present as a single copy on the *P. ananatis* LMG20103 genome.

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Two type IV pilin genes were selected for mutation. *pilA*, which encodes the major pilin subunit of the type IV pili (Kang *et al*., 2002; Bahar *et al*., 2009), was selected as it plays a crucial role in the synthesis of type IV pili (Semmler *et al*., 1999; Liu *et al*., 2001; Li *et al*., 2007). Type IV pili are, however, also involved in attachment, biofilm formation, virulence and horizontal gene transfer (HGT) (Soto and Hultgren, 1999; Rojas *et al*., 2002), so to differentiate between the effect that type IV pili have on the cell's general fitness and twitching motility specifically, a type IV pilin gene was selected that is involved in the motility of type IV pili. The selected gene was *pilT*. PilT belongs to a family of ATPases and is essential for retraction of type IV pili (Merz *et al*., 2000; Bahar *et al*., 2009; Burdman *et al*., 2011). Both *pilA* and *pilT* are present as single copy genes on the *P. ananatis* LMG20103 genome.

The size of the target genes can be found in Table 3.2 and their position in relation to neighbouring genes is demonstrated in Figure 3.1.

3.2.3 Generation of competent *P. ananatis* LMG20103 cells

Competent *P. ananatis* LMG20103 cells were generated to allow effective uptake of foreign DNA. In brief, cells from a pure culture of *P. ananatis* LMG20103 were inoculated into 10 ml LB broth with a sterile inoculating loop. The cultures were grown overnight at 28°C on a rotary shaker (150 rpm). 2 ml of the overnight culture was transferred to 100 ml SOC broth (10 mM $MgCl_2$, 2.5 mM KCl, 2% [w/v] tryptone, 0.5% [w/v] yeast extract, 0.05% [w/v] NaCl, 20 mM glucose), an outgrowth medium used to ensure maximum transformation efficiency. The cultures were incubated at 28°C with shaking rotation to an optical density (OD) reading of 0.6, (Molecular Devices SpectraMax Plus 384 spectrophotometer; wavelength (λ) = 600 nm). This ensured that the cells being used were in the exponential growth phase when the greatest number of viable cells are present. Sterile LB broth was used as a blank to zero the spectrophotometer. The cultures were transferred to 50 ml tubes and incubated on ice for 15 min and then centrifuged at 5000 rpm, 4°C, for 10 min (Eppendorf Centrifuge 5810 R). The pellet was washed with 200 µl ice-cold sterile dH_2O. The cells were centrifuged as before and washed twice with ice-cold 10% glycerol. The pellet was resuspended in 1 ml 10% glycerol and 50 µl aliquots were distributed into pre-chilled 0.2 ml tubes. The cells were snap-frozen in liquid nitrogen and stored at -70°C.

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3.2.4 Transformation with plasmid pRSFRedTER

Most bacteria have intracellular exonucleases that are capable of degrading foreign linear DNA (Datsenko and Wanner, 2000). Consequently, any integrative cassettes that are transformed into a bacterial cell will most likely be degraded. Bacteriophage λ has a λ Red system that encodes three genes coding for the Exo, Beta and Gam proteins. The Exo protein degrades double-stranded DNA in a 5′ – 3′ direction, producing 3′ single-stranded DNA tails, while Beta stably binds single-stranded DNA to its homologous counterpart. The Gam protein inhibits the exonucleases RecBCD and SbcCD, protecting the double-stranded DNA from degradation (Katashkina *et al*., 2009). It is thus advantageous to insert the λ Red system into a bacterium that is to be transformed with double stranded foreign DNA.

Pantoea ananatis LMG20103 was transformed with pRSFRedTER (obtained from J. I. Katashkina, Ajinomoto-Genetika Research Institute, Russia; Figure 3.2) to allow for efficient downstream transformations with double-stranded DNA. Plasmid pRSFRedTER is a broad-host range plasmid that is stably maintained in many Gram –ve and a few Gram +ve bacteria. It has a λ Red system under the control of a P_{lacUV5} promoter which is inducible with the use of IPTG. It also has a chloramphenicol resistance gene for selection purposes and a levansucrase gene which allows for easy curing of the plasmid from the host bacterium (Katashkina *et al*., 2009). Levansucrase synthesises levan polysaccharides in the presence of sucrose, which are toxic compounds and lethal to the host cell. By curing the cells of the plasmid, the mutant strains are able to grow without the additional metabolic burden of carrying another plasmid (Kaniga *et al*., 1991).

Competent *P. ananatis* LMG20103 cells were thawed on ice, and 2 µl (42 ng) of pRSFRedTER was added to 50 µl of bacterial inoculum. The transformation mixture was incubated on ice for 15 min and then transferred to a 0.1-cm electrode gap electroporation cuvette (EquiBio) and exposed to a single electrical pulse of 5 ms using an Eppendorf Multiporator® (2.5 kV and 200 Ω). Immediately following the electrical discharge, 1 ml of SOC broth was added to the cuvette. The cells were allowed to recover and express the phenotypic properties encoded on the plasmid for 4 h while being incubated at 28°C and 250 rpm.

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50 µl of the transformed cell suspension was spread onto LB agar amended with 50 µg/ml chloramphenicol, and incubated for 48 h at 28°C. The pBR322 plasmid (Fermentas) has a tetracycline resistance selective marker and was used as a positive control (cells were transformed with pBR322 as opposed to pRSFRedTER). These cells were spread onto LB agar containing 50 µg/ml tetracycline. Competent cells transformed with dH_2O were used as a negative control.

Single colonies of *P. ananatis* 20103(λ Red) were streaked out on fresh LB agar containing 50 µg/ml chloramphenicol. These plates were incubated overnight at 28°C.

3.2.5 Integrative cassette construction

- **Primer design**

Using PerlPrimer version 1.1.18 and BioEdit Sequence Alignment Editor version 7.1.3.0, primers were designed on the sequences directly upstream and downstream of each of the target genes (Table 3.3). Primers FF and FR annealed to the 5′ and 3′ end of the *flgK* gene, respectively. Primer F1RK is the reverse primer for FF and F2FK is the forward primer for FR. The positions of the primers in relation to the *flgK* gene are shown in Figure 3.3. On the 5′ ends of primers F1RK and F2FK, there is a 21 bp sequence that is homologous to the kanamycin resistance gene. Primers were similarly designed for *motA*, *pilA* and *pilT* (Table 3.3).

The kanamycin resistance gene was amplified from plasmid pKD13 (obtained from B. L. Wanner, Department of Biological Sciences, Purdue University, USA) using primers 13KanF and 13KanR (Table 3.3). A 21 bp sequence homologous to each target gene was added to the 5′end of 13KanF and 13KanR .

- **DNA extraction and PCR**

The wild-type strain *P. ananatis* LMG20103 was grown overnight in LB broth at 28°C, 250 rpm. Total genomic DNA was extracted using the ZR Genomic DNA II Kit™ (Zymo Research). The *Escherichia coli* K-12 strain harbouring pKD13 was grown overnight in LB broth at 36°C, 250 rpm. Plasmid DNA was extracted using the Zyppy™ Plasmid Miniprep Kit (Zymo Research) and stored at -20°C.

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The 5′- and 3′ ends of the target genes from the extracted *P. ananatis* LMG20103 DNA and the *kan* gene from pKD13 were amplified using the primers specified in Table 3.3. Each 25-µl PCR reaction mixture contained 10 × reaction buffer, 1.5 mM $MgCl_2$, 250 µM of each nucleotide (dATP, dGTP, dCTP, dTTP), 100 pmol of each primer (forward and reverse), 1 U of High Fidelity PCR Enzyme Mix (Fermentas) and 50-100 ng of DNA template. The High Fidelity PCR Enzyme Mix consists of thermostable *Taq* DNA polymerase with proofreading activity, ensuring the accuracy of the amplicon, as well as greater yields. The amplification was carried out in a Model 2720 thermal cycler (Applied Biosystems). The PCR conditions comprised of denaturation at 94°C for 5 min, 30 cycles of denaturation at 92°C for 1 min, annealing of primers at 66°C for 1 min, primer extension at 72°C for 3 min, and final chain elongation at 72°C for 5 min. A negative control (ddH_2O) was included with the PCR reactions to monitor for the presence of any contaminants. The amplicons were mixed with GelRed (Biotium, Hayward, California, USA) and electrophoresed on a 1% (w/v) agarose gel in 1 × TAE buffer (40 mM Tris acetate, 1 mM EDTA; pH 8.5) at 100 V for 30 min, and visualised and photographed under UV light.

- **Fusion PCR**

For each specified gene, the amplicons from the previous three PCRs (5′ gene fragment PCR, 3′ gene fragment PCR and *kan* PCR) were amalgamated in a fusion PCR designed to disrupt the selected genes. The PCR conditions were the same as stipulated above, except that the annealing temperature was set at 62°C. The amplicons were electrophoresed and visualised on an agarose gel.

- **Fusion PCR amplicon sequencing**

A band that corresponded to the correct size of each of the fusion products was identified on the gel (Figure 3.5). The *flgK* fusion product was expected to be 2,450 bp, the *motA* fusion product 1,900 bp, *pilA* fusion product 1 900 bp and *pilT* fusion product 1,742 bp. The bands were excised from the gel and purified using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research).

To verify that the correct bands had been excised from the gel, and that the selected genes were indeed disrupted with the *kan* gene, the purified amplicons were sequenced. The genes were sequenced in both a 5′→3′ and a 3′→5′ direction. The nucleotide sequence of the

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amplicons was determined using the ABI-PRISM® BigDye™ Terminator v.3.1 Cycle Sequencing Ready Reaction kit (Perkin-Elmer Applied Biosystems) according to the instructions of the manufacturer. The 10-µl sequencing reaction mixture contained BigDye™ (ABI PRISM®), 5 × sequencing buffer, nuclease-free (NF) H_2O, 10 pmol of primer and 100 ng of template DNA. The sequencing PCR reactions comprised of denaturation at 96°C for 5 s, and 25 cycles of denaturation at 96°C for 10 s, annealing of primers at 55°C for 5 s, and primer extension at 60°C for 4 min. The extension products were precipitated by adding 16 µl of ice-cold absolute EtOH and 2 µl of 3 M NaOAc (pH 4.8). The precipitated DNA was washed twice in 70% EtOH before being pelleted again and dried. The samples were sequenced on an Applied Biosystems ABI3500xl sequencer at the University of Pretoria's DNA Sequencing Core Facility.

3.2.6 Transformation with integrative cassettes

P. ananatis 20103(λ Red) cells were made competent following the protocol stipulated in Section 3.2.3. The SOC broth was amended with 50 µg/ml chloramphenicol and 2 mM IPTG. The addition of IPTG was necessary to induce the P_{lac} of pRSFRedTER so that the *gam*, *beta* and *exo* genes would be expressed (Katashkina *et al*., 2009).

Competent *P. ananatis* 20103(λRed) cells were transformed with each of the four fusion cassettes respectively, following the protocol stipulated in Section 3.2.4. The cells were spread onto LB agar containing 50 µg/ml kanamycin, and incubated for 24 h at 28°C. pBR322 was used as a positive control and transformation with ddH_2O was used as a negative control. Single colonies were selected from the 50 µg/ml kanamycin plates and streaked out onto fresh LB agar that had been amended with 50 µg/ml kanamycin, and incubated overnight at 28°C. The resulting four strains were used for all further experiments and were named PA(*flgK*$^-$), PA(*motA*$^-$), PA(*pilA*$^-$) and PA(*pilT*$^-$).

3.2.7 Plasmid curing

To prevent spontaneous mutations from occurring at a later stage, pRSFRedTER was evicted from the four mutant strains. This was done by growing strains PA(*flgK*$^-$), PA(*motA*$^-$), PA(*pilA*$^-$) and PA(*pilT*$^-$) on LB agar amended with 10% sucrose (Merck). Strains grown on sucrose-amended LB agar were repli-plated on both 50 µg/ml kanamycin and 50 µg/ml

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chloramphenicol plates. Strains that were able to grow on kanamycin but were unable to grow on chloramphenicol were selected for further studies.

3.2.8 Mutant verification PCRs

Total genomic DNA was extracted from PA(*flgK*$^-$), PA(*motA*$^-$), PA(*pilA*$^-$) and PA(*pilT*$^-$) using the ZR Genomic DNA II Kit™. The extracted DNA was stored at -20°C. The disruption of each of the four target genes with the *kan* gene was verified by conducting a PCR whereby the forward primer (known as the test primer) annealed to a region upstream of the target gene (Table 3.4) and the reverse primer would bind to the *kan* gene. Following the PCR protocol in Section 3.2.5, the verification regions were amplified and visualised.

3.2.9 Southern blot hybridisation

A Southern blot hybridisation (Southern, 1975) was performed to determine whether the *kan* gene had only been inserted once into the genome of each of the four mutant strains. This was done to ensure that any changes in the phenotype of the mutant strains could be attributed to the disruption of the genes *flgK*, *motA*, *pilA* and *pilT*, respectively.

The *kan* gene amplified from the extracted pKD13 from *E. coli* K1-12 was used as a probe. The gene was labelled with a DIG DNA Labelling and Detection Kit (Roche Diagnostics) according to the manufacturer's instructions, and the labelling efficiency of the probe was tested. Chromosomal DNA extracted from *P. ananatis* strains PA(*flgK*$^-$), PA(*motA*$^-$), PA(*pilA*$^-$) and PA(*pilT*$^-$) as well as the wild-type strain LMG20103, was digested with the restriction enzymes *Xho*I, *Cla*I and *Ase*I. The digested DNA was electrophoresed on a 1% agarose gel with the unlabelled probe (*kan* gene) serving as a positive control. The DNA was then transferred from the agarose gel to a nylon membrane (Hybond™-N; Amersham-Pharmacia Biotech) using the stacking method. The DNA was fixed to the membrane by UV irradiation for 5 min and then hybridized overnight at 65°C. The hybridised DNA was detected immunologically with alkaline phosphatase-conjugated anti-digoxigenin antibodies (Roche Diagnostics).

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3.2.10 Complementations

Strains PA(*flgK*$^-$), PA(*motA*$^-$), PA(*pilA*$^-$) and PA(*pilT*$^-$) were complemented to ensure that any change in the phenotype of the mutants was due to the disruption of the selected genes. It was expected that the phenotype of the complemented strains would be indistinguishable from that of the wild type strain. For each mutant, two complements were made, one with the gene of interest under the control of a *lac* promoter and another under the control of the selected gene's native promoter. The mutants were complemented with their target gene under the control of its native promoter as it would ensure that the selected gene would have the same expression levels as the wild type strain. Genes under the control of a *lac* promoter may be over-expressed and are therefore not comparable to the wild-type strain. However, *lac* promoter complements were included as they are known to have a high degree of success in complementation studies. The *lac* promoter complements were made available for use should the native promoter complementations produce unsubstantial amounts of the targeted proteins.

- **Adding restriction enzyme cut sites to the selected genes**

To complement the mutants, functional copies of the selected genes were needed. For the *lac* promoter complements, primers were designed so that the forward and reverse primers annealed to the DNA sequence directly adjacent to the selected genes. Functional copies of the neighbouring genes or any promoter sequences were excluded. To ensure the correct orientation of the genes, different restriction enzyme cut sites were added to the ends of the target genes. The restriction enzyme cut site for *Bam*HI was added to the 5′ end of the forward primer with a GC clamp, and the *Xho*I cut site and a GC clamp was added to the 3′ end of the reverse primer. A GC clamp adds stability to the enzyme digestions as it allows the enzyme to bind to the DNA with greater ease. The primer sequences for all the selected gene complementations are included in Table 3.5.

The primers for the native promoter complements were designed to include the native promoter for each of the target genes (Table 3.5). *Xho*I cut sites with a GC clamp were added to the 5′ end of the forward primer and 3′ end of the reverse primer. To ensure that the native promoters were included, the promoter sequences were first located in the upstream sequences. The *flgK* and *motA* genes have σ^{28} (RpoF) promoters, whereas *pilA* and *pilT* have σ^{70} (RpoD) promoters. Sigma factors are bacterial transcription initiation factors that enable

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RNA polymerases to bind to specific gene promoters (Madigan *et al*., 2003). The upstream sequences of *flgK* and *motA* were manually searched for their promoter sequences. The σ^{70} promoters of *pilA* and *pilT* were predicted using BPROM (Solovyev and Salamov, 2011). BPROM predicts potential transcription start sites in bacterial genomes that are regulated by σ^{70} promoters. For both *pilA* and *pilT*, 200 bp of sequence upstream of the gene was analysed as most promoters are found within 150 bp of the corresponding gene (Madigan *et al*., 2003). The eight target regions were thus amplified using the primers in Table 3.5.

- **Plasmid extraction**

The plasmid pBBR1MCS-5 was selected for complementation of the mutants (Figure 3.4). pBBR1MCS-5 has a gentamicin resistance gene, a *lac* promoter and a multiple cloning site (Kovach *et al*., 1995). pBBR1MCS-5 was extracted from *E. coli* K-12 using the Zyppy™ Plasmid Miniprep Kit and the extracted DNA was stored at -20°C.

- **Digestion and ligation**

The gene amplicons under the control of the *lac* promoter were digested with *Xho*I and *Bam*HI, whereas those with native promoters were digested with *Xho*I only. Digests were performed by adding 5 µl of amplified DNA to 2 µl restriction enzyme buffer and 5 units of enzyme. NF H_2O was added to a total volume of 20 µl and incubated overnight at 37°C. The digested DNA was purified using the DNA Clean & Concentrator™ kit (Zymo Research).

In two separate reactions pBBR1MCS-5 was digested with *Xho*I and *Bam*HI, and *Xho*I, respectively. 5 µg of plasmid DNA was added to 2 µl restriction enzyme buffer and 5 units of enzyme. NF H_2O was added to a total volume of 20 µl and incubated overnight at 37°C. The digested DNA was purified and visualised on a 1% agarose gel (Figure 3.8).

Digested amplicons and plasmid DNA were ligated by adding 1 µl of the digested pBBRM1CS5 to 2 µl of the digested target gene, 1 µl 10× buffer, 5 µl NF H_2O and 1 µl T4 DNA ligase (Roche Diagnostics). The ligations were incubated overnight at 14°C, and an aliquot of the resulting products was electrophoresed on a 1% agarose gel.

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- **Transformations**

To complement the mutations, each of the four mutant *P. ananatis* strains were made competent as described in Section 3.2.3. Competent cells were thawed on ice, and 2 µl (40 ng) of pBBRM1CS-5 ligated with the respective PCR amplicons was added to 50 µl of cells. The transformation mixture was electroporated into the competent cells where after the cells were allowed to recover for 4 h while being incubated at 28°C and 250 rpm.

The cells were then spread onto LB agar containing 50 µg/ml gentamicin, and incubated for 48 h at 28°C. The mutant strains PA(*flgK*$^-$), PA(*motA*$^-$), PA(*pilA*$^-$) and PA(*pilT*$^-$) were also spread on LB agar containing 50 µg/ml gentamicin as a negative control. pBR322 was included as a positive control. All plates were incubated overnight at 28°C.

Single colonies from the eight complemented *P. ananatis* strains were selected and streaked out on fresh LB agar containing 50 µg/ml gentamicin. These plates were incubated overnight at 28°C.

3.2.11 Complementation verification

To verify that the mutant strains had been complemented, pBBRM1CS-5 was extracted from each of the eight complemented strains using the Zyppy^{TM} Plasmid Miniprep Kit. Using the primers described in Table 3.5, PCR amplifications were conducted to verify the presence of the respective genes. The PCR amplicons were subsequently sequenced in both a forward and reverse direction.

To ensure that the four complementations that were under the control of a *lac* promoter were present in the correct orientation, further PCR amplifications were conducted. This was imperative as the *lac* promoter would be unable to initiate transcription of a gene that was in the incorrect orientation. The forward primer (5´-CCTTTCGCTATTACGCCAGCTG-3´) used in each PCR reaction annealed upstream of the multiple cloning site in pBBRM1CS-5, with the target gene reverse primers listed in Table 3.5. The correct orientation of the gene would thus result in an amplicon whereas no amplification would result for a gene in the incorrect orientation.

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3.2.12 Growth assays

To ensure that the mutations and complementations did not adversely affect the growth of the transformed strains, growth assays were conducted and compared to that of the wild type strain, *P. ananatis* LMG20103. The strains were cultured overnight and 1 ml of the overnight culture was inoculated into 50 ml fresh LB broth. The wild-type, mutant and complemented strains were grown until they reached an OD_{600} of 0.6. 1 ml of the cultures was again inoculated into fresh LB broth, and spectrophotometric readings (λ = 600 nm) were taken every hour for 14 h. Sterile LB broth was used as a blank. The strains were grown at 28°C in a rotary shaker set at 250 rpm for the duration of the experiment. The growth assays were conducted in triplicate. A logarithmic scale of the average absorbances was plotted against the time frame of the experiment.

3.3 RESULTS AND DISCUSSION

3.3.1 Antibiotic sensitivity assays identify antibiotic selective markers for mutagenesis

P. ananatis LMG20103 is susceptible to kanamycin, tetracycline, gentamicin and chloramphenicol, whereas no zones of inhibition were observed for several of the other tested antibiotics including neomycin, penicillin G and erythromycin (Tables 3.6 and 3.7). Based on these results, kanamycin, tetracycline, chloramphenicol and gentamicin were identified as being efficient selective markers for transformation or mutagenesis of *P. ananatis* LMG20103.

3.3.2 Integrative cassette construction and verification

Through homologous recombination and the aid of the λ Red system, the *kan* gene was integrated into the *flgK*, *motA*, *pilA* and *pilT* genes in strains PA(*flgK*$^-$), PA(*motA*$^-$), PA(*pilA*$^-$) and PA(*pilT*$^-$), respectively. This was confirmed by PCR amplification and the length of the fusion PCR amplicon obtained (Figure 3.5). Sanger sequencing of the amplicon from both the 5′ and 3′ ends further confirmed the integration of the *kan* gene within the target genes (data not shown). The strains transformed with the integrative cassettes grew on LB agar plates amended with kanamycin whereas the wild-type strain could not.

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To verify the correct localisation of the integrated cassettes in the genomes of strains PA(*flgK*$^-$), PA(*motA*$^-$), PA(*pilA*$^-$) and PA(*pilT*$^-$), mutation verification PCRs were conducted as stipulated in Section 3.2.8. The resulting amplicons can be seen in Figure 3.6. The Southern blot hybridisation demonstrated that the *kan* gene was only inserted into the genomes of strains PA(*flgK*$^-$), PA(*motA*$^-$), PA(*pilA*$^-$) and PA(*pilT*$^-$) once (Figure 3.7). A *kan*-specific probe was used to detect the presence of the *kan* gene in chromosomal DNA extracted from both the wild-type and mutant strains. The probe was unable to bind to the wild-type DNA as it does not have a copy of the *kan* gene.

3.3.3 Plasmid curing

It was assumed that the colonies of PA(*flgK*$^-$), PA(*motA*$^-$), PA(*pilA*$^-$) and PA(*pilT*$^-$) that grew on media amended with 10% sucrose had been cured of pRSFRedTER. This was confirmed by repli-plating the strain onto media amended with kanamycin and chloramphenicol. Strains that were unable to grow in the presence of chloramphenicol had lost pRSFRedTER and its plasmid-borne chloramphenicol resistance gene.

3.3.4 Complementation of the mutant strains

The complemented mutant strains were able to grow on LB plates amended with gentamicin which is encoded on pBBR1MCS-5, and showed the typical growth and morphology of the wild-type strain on LB plates. The wild-type and mutant strains displayed no growth on LB amended with gentamicin. pBBR1MCS-5 was extracted from all eight complemented strains and subjected to PCR to confirm the presence of the *flgK*, *motA*, *pilA* and *pilT* genes, respectively (Figure 3.9). These genes were also Sanger sequenced (data not shown) to ensure that each copy of the gene was intact. The *lac* promoter complemented strains were scrutinised further to ensure the correct orientation of the genes under the control of the *lac* promoter (Figure 3.10).

3.3.5 Mutant and complemented strains display similar growth patterns as the wild-type strain

For further functional analysis of the targeted genes, it is imperative that the mutations do not have a deleterious effect on the growth of the knock-out mutants. Similarly, the growth of the complemented strains should not be impeded. Growth assays were performed for each of the four mutant strains, the eight complemented strains and the wild-type strain. Similar growth

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patterns were observed for both the wild-type and mutant strains (Figure 3.11). Likewise, the growth of the four complemented strains with a *lac* promoter (Figure 3.12) and a native promoter (Figure 3.13), were similar to that of the wild-type strain. The genotypic alterations of the mutant and complemented strains did thus not impede the growth of the mutant and complemented strains.

3.4 CONCLUSIONS

The recent development of novel site-directed mutagenesis circumvents many of the pitfalls associated with standard random mutagenesis methodologies which are laborious and time-consuming. Here we have applied genetic recombineering to construct four *P. ananatis* motility mutants. Recombineering involves the transformation of the host strain with the phage λ Red system prior to mutagenesis as it allows foreign DNA to enter the cell without being degraded, and aids homologous recombination. Two flagellar mutants were created by disrupting the *flgK* and *motA* genes with a kanamycin resistance gene. FlgK is a hook-associated protein that is essential for the assembly of flagella whereas MotA forms part of the motor and is required for flagellar rotation. Both these mutations would prevent swimming motility by incapacitating the flagella. Two twitching motility mutants were generated by impairing the structure and function of type IV pili. Both the gene encoding the major pilin subunit, *pilA*, and the gene encoding the twitching motility ATPase, *pilT*, were disrupted with a kanamycin resistance gene. Furthermore, each strain was complemented twice; once with the target gene under control of a *lac* promoter and once with the gene under the control of its native promoter. Native promoters were included in this study to ensure comparable expression levels of the gene of interest. These mutants and complemented strains will be applied to study the role of swimming and twitching motility in the infection process of *P. ananatis on onion.*

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FIGURES

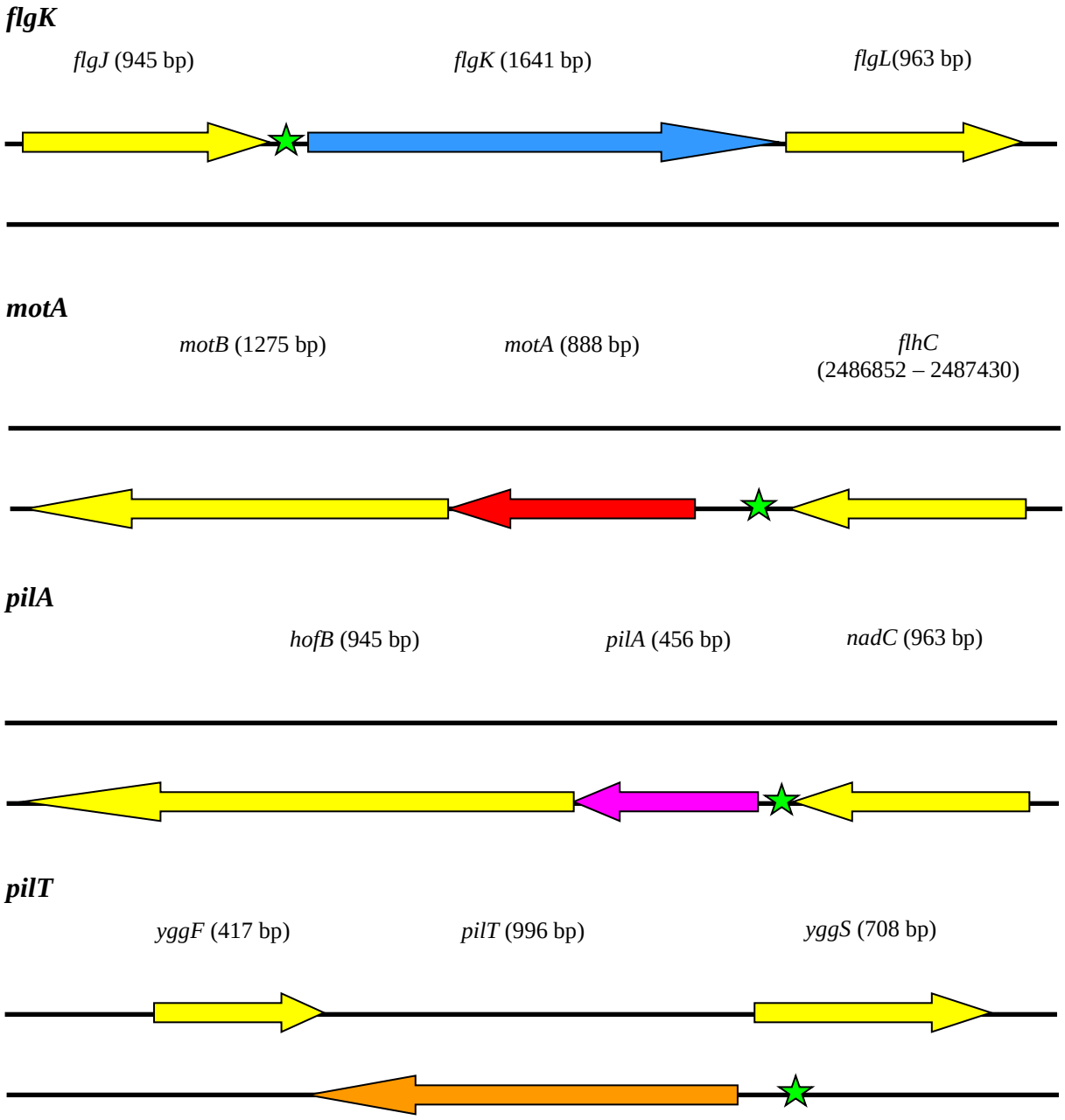

Figure 3.1: The position of the genes selected for disruption in the *P. ananatis* LMG20103 genome in relation to their upstream and downstream flanking genes. The numbers in brackets indicate the size of the target genes and the green stars are indicative of the position of the promoter of each gene.

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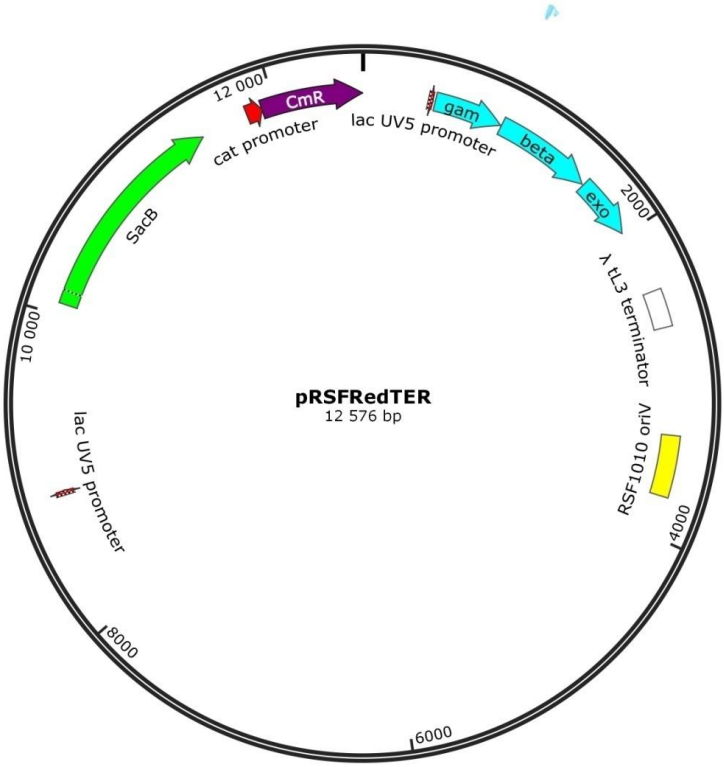

Figure 3.2: Diagramatic representation of pRSFRedTER. P_{lacUV5} is an inducible *lac* promoter and *sacB* encodes levansucrase which renders bacterial growth sensitive to sucrose. Chloramphenicol resistance is encoded by the Cm^R gene which is under the control of the *cat* promoter. Genes *gam*, *beta* and *exo* make up the λ Red phage system. Transcription terminates at the λtL3 terminator (Katashkina *et al*., 2009).

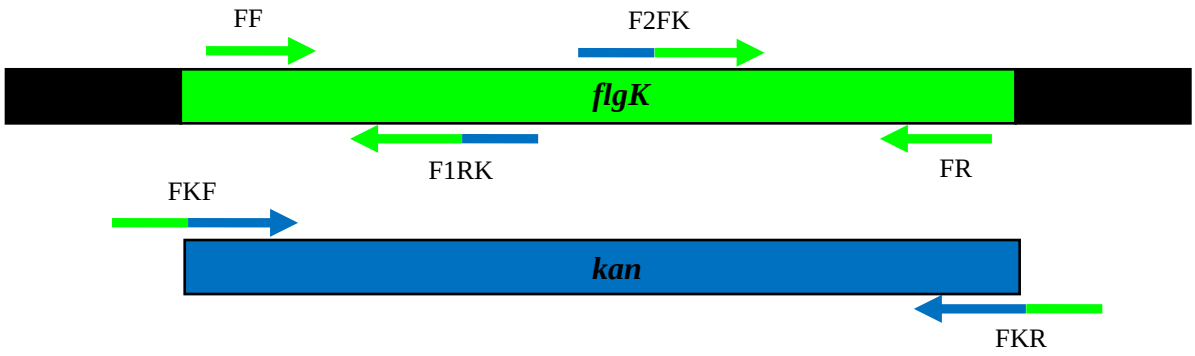

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Figure 3.3: Diagram depicting primer annealing sites to the target and kanamycin resistance genes. Diagram is not drawn to scale. The blue bars indicate the primer sequences that are homologous to the kanamycin resistance gene. The green bars indicate the primer sequences that are homologues to the *flgK* gene.

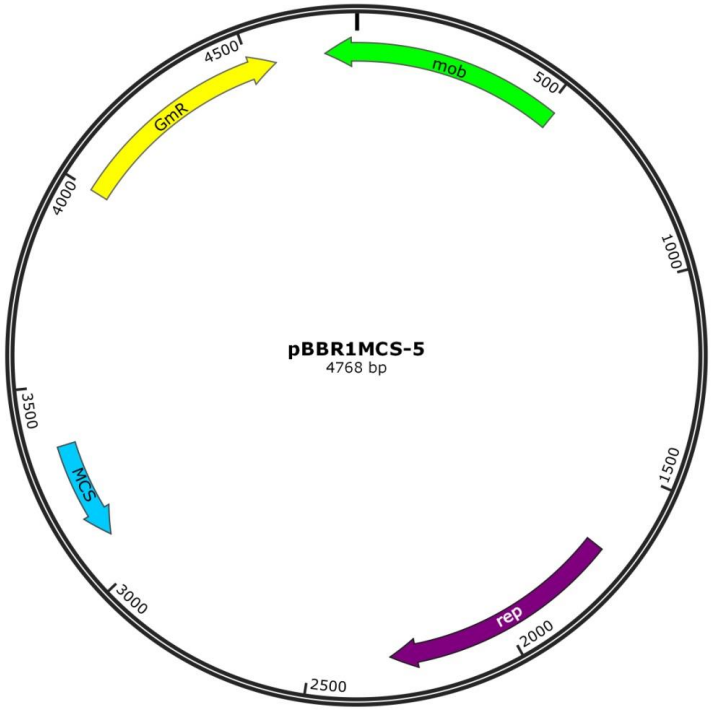

Figure 3.4: Diagrammatic representation of the pBBR1MCS-5 plasmid. pBBR1MCS-5 was used to complement the mutants. Gm^R denotes the gentamicin resistance gene, MCS is the multiple cloning sites where the unique restriction enzyme cut sites are located, *mob* is a mobile element that allows for transformation and *rep* is the origin of replication (Kovach *et al*., 1995). The genes used to complement the mutants were inserted into the MCS.

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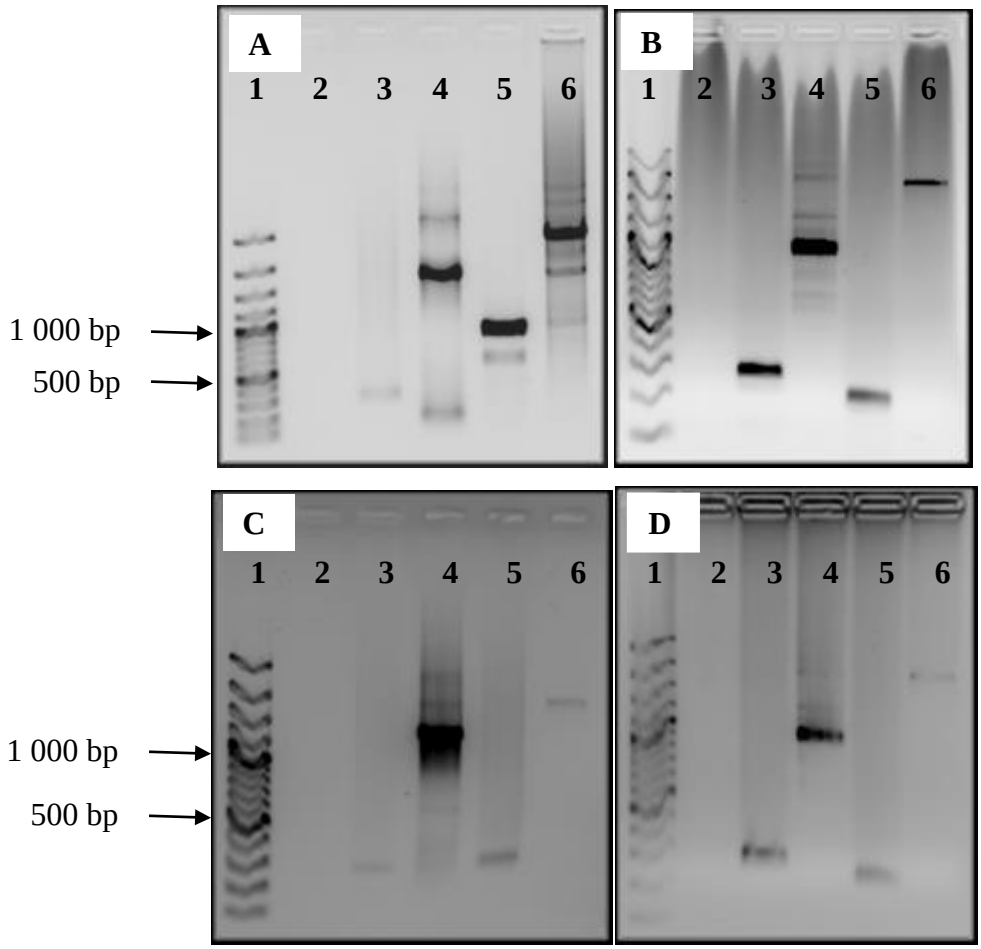

Figure 3.5: Gel images of the fusion PCR integrative cassettes. In all four gels the following was loaded onto the gel; lane 1: 100 bp ladder, lane 2: negative control, lane 3: 5´ amplicon of target gene, lane 4: *kan* gene, lane 5: 3´ amplicon of target gene, lane 6: fusion PCR product, i.e. integrative casette. Gel A shows the *flgK* integrative cassette, gel B the *motA* integrative cassette, gel C the *pilA* integrative cassette and gel D the *pilT* integrative cassette.

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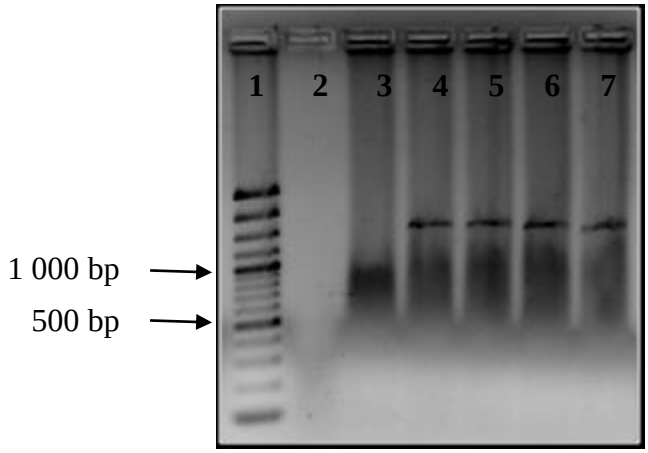

Figure 3.6: Gel images of the mutant verification PCRs. Lane 1: 100 bp ladder, lane 2: negative control, lane 3: *P. ananatis* LMG20103 DNA amplified with primers TestFF and 13KanR, lane 4: PA(*flgK*$^{-}$) DNA amplified with primers TestFF and 13KanR, lane 5: PA(*motA*$^{-}$) DNA amplified with primers TestMF and 13KanR, lane 6: PA(*pilA*$^{-}$) DNA amplified with primers TestPF and 13KanR, lane 7: PA(*pilT*$^{-}$) DNA amplified with primers TestTF and 13KanR.

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Figure 3.7: Southern blot to detect the *kan* gene in *P. ananatis* LMG20103 and the four mutants. Lane 1: *P. ananatis* LMG20103 chromosomal DNA, lane 2: chromosomal DNA of *P. ananatis* strain PA(*flgK*$^{-}$), lane 3: chromosomal DNA of *P. ananatis* strain PA(*motA*$^{-}$), lane 4: chromosomal DNA of *P. ananatis* strain PA(*pilA*$^{-}$), lane 5: chromosomal DNA of *P. ananatis* strain PA(*pilT*$^{-}$), lane 6: positive control (*kan* gene). All DNA was digested with *Xho*I, *Cla*I and *Ase*I.

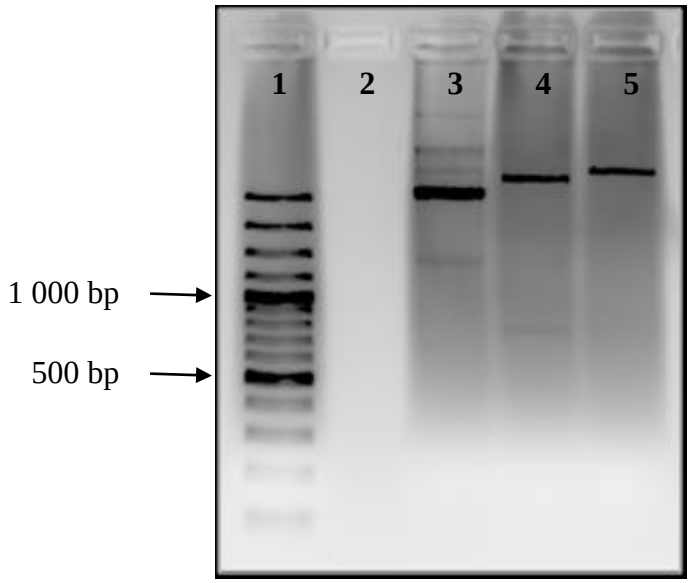

Figure 3.8: Gel image of the digested pBBR1MCS-5. Lane 1: 100 bp ladder, lane 2: negative control, lane 3: undigested pBBR1MCS-5, lane 4: pBBR1MCS-5 digested with *Bam*HI and *Xho*I, lane 5: pBBR1MCS-5 digested with *Xho*I only.

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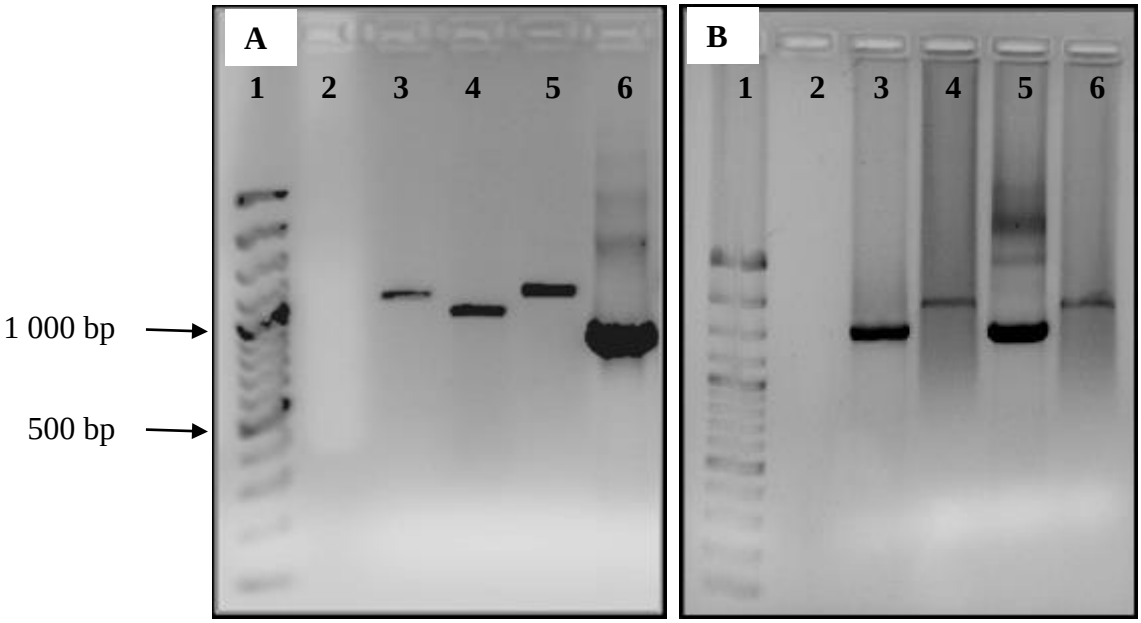

Figure 3.9: PCR verification of complemented mutants. (A) Gel image of the amplicons from the complemented strains that are under the control of the *lac* promoter. Lane 1: 100 bp ladder, lane 2: negative control, lane 3: CpFlgK, lane 4: CpMotA, lane 5: CpPilA, lane 6: CpPilT. (B) Gel image of the amplicons from the complemented strains that are under the control of the target gene's native promoter. Lane 1: 100 bp ladder, lane 2: negative control, lane 3: CnFlgK, lane 4: CnMotA, lane 5: CnPilA, lane 6: CnPilT.

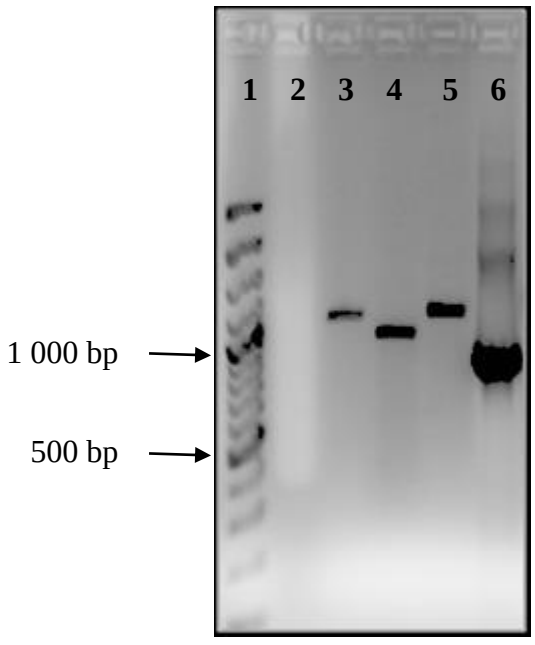

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Figure 3.10: Gel image to verify the correct orientation of the genes under the control of the *lac* promoter. Lane 1: 100 bp ladder, lane 2: negative control, lane 3: CpFlgK, lane 4: CpMotA, lane 5: CpPilT, lane 6: CpPilA.

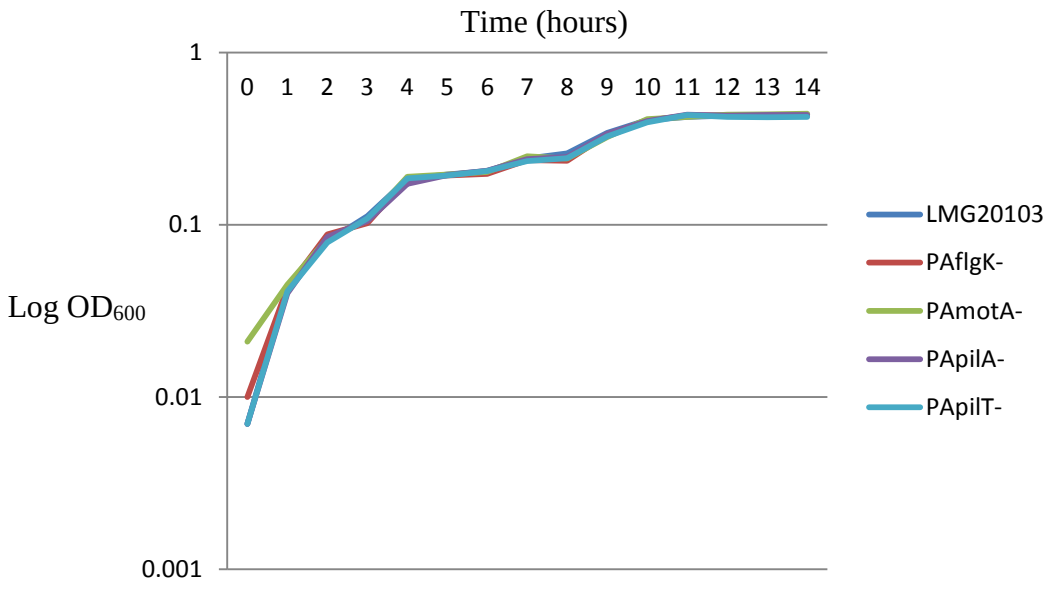

Figure 3.11: Growth curves of the four mutant strains in comparison with the wild type strain, *P. ananatis* LMG20103, over a period of 14 hours.

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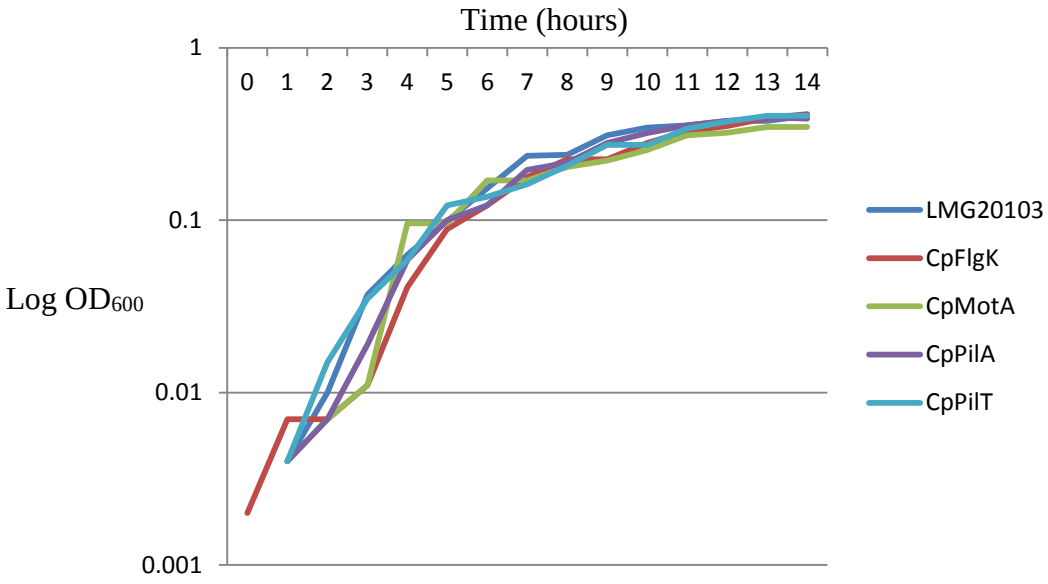

Figure 3.12: Growth curves of the four *lac* promoter complemented strains in comparison with the wild type strain, *P. ananatis* LMG20103, over a period of 14 hours.

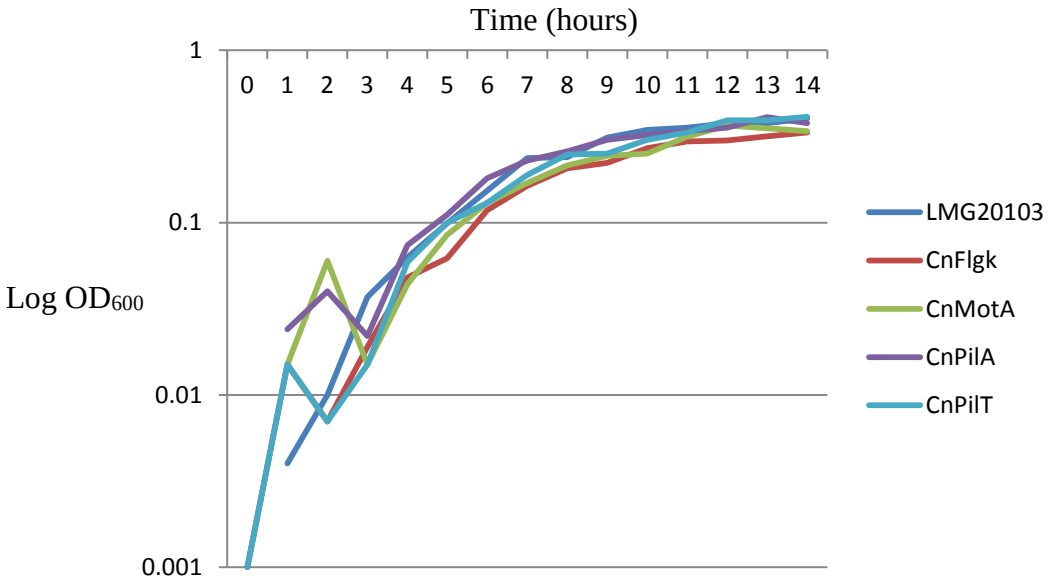

Figure 3.13: Growth curves of the four native promoter complemented strains in comparison with the wild type strain, *P. ananatis* LMG20103, over a period of 14 hours.

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TABLES

Table 3.1: Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics[a]
Strains	
Pantoea ananatis	
LMG20103	Wild-type, virulent *Euaclyptus* isolate; Swm^+, Twt^+
20103(λ Red)	Wild-type carrying pRSFRedTER; Cm^r
PA(*flgK*$^-$)	LMG20103 deletion mutant defective in *flgK*; Kan^r, Swm^-, Twt^+
PA(*motA*$^-$)	LMG20103 deletion mutant defective in *motA*; Kan^r, Swm^-, Twt^+
PA(*pilA*$^-$)	LMG20103 deletion mutant defective in *pilA*; Kan^r, Swm^+, Twt^-
PA(*pilT*$^-$)	LMG20103 deletion mutant defective in *pilT*; Kan^r, Swm^+, Twt^-
PA(*flgK*$^-$) (λ Red)	PA(*flgK*$^-$) carrying pRSFRedTER; Kan^r, Cm^r
PA(*motA*$^-$) (λ Red)	PA(*motA*$^-$) carrying pRSFRedTER; Kan^r, Cm^r
PA(*pilA*$^-$) (λ Red)	PA(*pilA*$^-$) carrying pRSFRedTER; Kan^r, Cm^r
PA(*pilT*$^-$) (λ Red)	PA(*pilT*$^-$) carrying pRSFRedTER; Kan^r, Cm^r
PA(*flgK*$^-$)-laccomp	PA(*flgK*$^-$) complemented with pBBRMCS-5 (using *lac* promoter); Gm^r, Kan^r
PA(*flgK*$^-$)-natcomp	PA(*flgK*$^-$) complemented with pBBRMCS-5 (using native promoter); Gm^r, Kan^r
PA(*motA*$^-$) -laccomp	PA(*motA*$^-$) complemented with pBBRMCS-5 (using *lac* promoter); Gm^r, Kan^r
PA(*motA*$^-$) -natcomp	PA(*motA*$^-$) complemented with pBBRMCS-5 (using native promoter); Gm^r, Kan^r
PA(*pilA*$^-$) -laccomp	PA(*pilA*$^-$) complemented with pBBRMCS-5 (using *lac* promoter); Gm^r, Kan^r
PA(*pilA*$^-$) -natcomp	PA(*pilA*$^-$) complemented with pBBRMCS-5 (using native promoter); Gm^r, Kan^r
PA(*pilT*$^-$) -laccomp	PA(*pilT*$^-$) complemented with pBBRMCS-5 (using *lac* promoter); Gm^r, Kan^r
PA(*pilT*$^-$) -natcomp	PA(*pilT*$^-$) complemented with pBBRMCS-5 (using native promoter); Gm^r, Kan^r
Plasmids	
pRSFRedTER	Vector containing λ Red system and levansucrase gene;

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	Cm^r
pKD13	Kan^r
pBBR1MCS-5	Complementation vector; Gm^r, *lac* promoter
pBBR1MCS-5- PA(*flgK*$^-$) -LP	pBBRM1CS-5 with the 1.641 kb *flgK* gene cloned into the *Bam*HI and *Xho*I sites downstream of the *lac* promoter
pBBR1MCS-5- PA(*motA*$^-$) -LP	pBBRM1CS-5 with the 888 bp *motA* gene cloned into the *Bam*HI and *Xho*I sites downstream of the *lac* promoter
pBBR1MCS-5- PA(*pilA*$^-$) -LP	pBBRM1CS-5 with the 456 bp *pilA* gene cloned into the *Bam*HI and *Xho*I sites downstream of the *lac* promoter
pBBR1MCS-5- PA(*pilT*$^-$) -LP	pBBRM1CS-5 with the 996 bp *pilT* gene cloned into the *Bam*HI and *Xho*I sites downstream of the *lac* promoter
pBBR1MCS-5- PA(*flgK*$^-$) -NP	pBBRM1CS-5 with the 1.841 kb *flgK* and native promoter cloned into the *Xho*I site
pBBR1MCS-5- PA(*motA*$^-$) -NP	pBBRM1CS-5 with the 1.088 kb *motA* and native promoter cloned into the *Xho*I site
pBBR1MCS-5- PA(*pilA*$^-$) -NP	pBBRM1CS-5 with the 656 bp *pilA* and native promoter cloned into the *Xho*I site
pBBR1MCS-5- PA(*pilT*$^-$) -NP	pBBRM1CS-5 with the 1.196 kb *pilT* and native promoter cloned into the *Xho*I site

[a] Swm: swimming motility; Twt: twitching motility; Kan^r, Gm^r: resistance to kanamycin and gentamicin, respectively

Table 3.2: The lengths of the genes selected for the generation of motility mutants in *P. ananatis* LMG20103

Selected Genes	Length (bp)
flgK	1641
motA	888
pilA	456
pilT	996

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Table 3.3: Primers used to construct integrative cassettes for deletion mutations

Primer*	Sequence (5´- 3´)
FF	GCATAATTAACTCCGCAATGAG
F1RK	AGCTCCAGCCTACACAATCGCTGAGCCGCGATAAAACTATC
F2FK	GGTCGACGGATCCCCGGAATCGCGTTTTAACGCCGTGCATAAG
FKF	GATAGTTTTATCGCGGCTCAGCGATTGTGTAGGCTGGAGCT
FKR	CTTATGCACGGCGTTAAAACGCGATTCCGGGGATCCGTCGACC
FR	CGCCATGTAATATTGTTGG
MF	CAACGGTAAATCGATCAAGAAGAC
M1RK	AGCTCCAGCCTACACAATCGCGCATTCATGTTGCCACTGACC
M2FK	GGTCGACGGATCCCCGGAATACGTCAGAAATGTGCAGAAA
MKF	GGTCAGTGGCAACATGAATGCGCGATTGTGTAGGCTGGAGCT
MKR	TTTCTGCACATTTCTGACGTATTCCGGGGATCCGTCGACC
MR	GATCCGAAGTCTGTTTAGCC
PF	CTGGTCGAGTTAATGATTGTGG
P1RK	AGCTCCAGCCTACACAATCGCGCGGAATCCCTCGCAGCCCGG
P2FK	GGTCGACGGATCCCCGGAATCGCTCAGCGGTCACCGTCGTGAA
PKF	CCGGGCTGCGAGGGATTCCGCGCGATTGTGTAGGCTGGAGCT
PKR	TTCACGACGGTGACCGCTGAGCGATTCCGGGGATCCGTCGACC
PR	TCAGACGTGGGCATAGGCAC
TF	ATGAATTAGTGGAGCTTAGTGT
T1RK	AGCTCCAGCCTACACAATCGCAGGCGAAATCCAACTGCCCT
T2FK	GGTCGACGGATCCCCGGAATCGCGGCCACAGATGAGACACGCGTGG
TKF	AGGGCAGTTGGATTTCGCCTGCGATTGTGTAGGCTGGAGCT
TKR	CCACGCGTGTCTCATCTGTGGCCGCGATTCCGGGGATCCGTCGACC
TR	TAACCGACTCTGCTCAAAGG
13KanF	GCGATTGTGTAGGCTGGAGCT
13KanR	ATTCCGGGGATCCGTCGACC

*F = *flgK*, M = *motA*, P = *pilA*, T = *pilT*

Table 3.4: Primers used to verify the mutations in strains PA(*flgK*$^-$), PA(*motA*$^-$), PA(*pilA*$^-$) and PA(*pilT*$^-$)

Primer*	Sequence (5´- 3´)
13KanR	ATTCCGGGGATCCGTCGACC
TestFF	AGCCGAGCTACAACGTCTTCGG
TestMF	CCACCTAAAGGCATGTTGCCTT
TestPF	CCGTCTGGGTCTGTCAGATGCC
TestTF	GCGCGCGCTGGTTTGTTTGAAC

*F = *flgK*, M = *motA*, P = *pilA*, T = *pilT*

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Table 3.5: Primers used to complement the mutated strains PA(*flgK*$^-$), PA(*motA*$^-$), PA(*pilA*$^-$) and PA(*pilT*$^-$)

Primer*	Sequence (5´- 3´)
CLFF	CGCCTCGAGTAACAGCAAGAGCCAGGCGC
CLFR	CGCGGATCCTCGGTAAAGGTCTTGTATGAAGCCTGCAG
CLMF	CGCCTCGAGGGTTAAACACGCCGTTTAAA
CLMR	CGCGGATCCCTTGTGTGTCAGTTTCGTTTTAC
CLPF	CGCCTCGAGCTTCTTATTCAGGAGGTAAT
CLPR	CGCGGATCCTACAGGAAGATCTTGGCGGTGAGGTAGACAGC
CLTF	CGCCTCGAGCGGACTTGCTGTAAGTTGTG
CLTR	CGCGGATCCCCATAGCCCAAATCGAAAGC
CNFF	CGCCTCGAGCAAGCCGAGCTACAACGTCTTCGGCATTAAAG
CNFR	CGCCTCGAGTCGGTAAAGGTCTTGTATGAAGCCTGCAG
CNMF	CGCCTCGAGCATATAAGGTTCAACTTCTGCGCTG
CNMR	CGCCTCGAGCTTGTGTGTCAGTTTCGTTTTAC
CNPF	CGCCTCGAGGTATCAAACTGCAGGGGGAGAGGATGCAGGAC
CNPR	CGCCTCGAGTACAGGAAGATCTTGGCGGTGAGGTAGACAGC
CNTF	CGCCTCGAGGACGATCGACCGTATGACAC
CNTR	CGCCTCGAGCCATAGCCCAAATCGAAAGC

*F = *flgK*, M = *motA*, P = *pilA*, T = *pilT*. Primers prefaced CL denote primers for genes that are under the control of a *lac* promoter whereas primers prefaced with CN denote primers for genes that are under the control of a native promoter. Underlined sequences indicate restriction enzyme binding sites.

Table 3.6: Results of *P. ananatis* LMG20103 antibiotic sensitivity assays using filter paper discs

Antibiotic	Concentration (µg/ml)	Diameter of zone of inhibition (mm ± standard deviation)
Gentamycin	40	24 ± 1.5
Tetracycline	30	27 ± 2.1
Ampicillin	100	21 ± 1.5
Kanamycin	50	28 ± 0.6
Streptomycin	50	16 ± 1.7
Neomycin	50	0

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Table 3.7: Results of *P. ananatis* LMG20103 antibiotic sensitivity assays using antibiograms

Abbreviation	Antibiotic	Concentration (µg/ml)	Diameter of zone of inhibition (mm ± standard deviation)
Mastring-S M11			
C	Chloramphenicol	25	22 ± 2.6
E	Erythromycin	5	0
FC	Fusidic Acid	10	0
MT	Methicillin	10	0
NO	Novobiocin	5	0
PG	Penicillin G	1 Unit	0
S	Streptomycin	10	12 ± 1.5
T	Tetracycline	25	25 ± 2.9
Microring TRA1			
Amk	Amikacin	10	21 ± 0.6
Czl	Cefazolin	30	0
Ery	Erythromycin	15	0
Lin	Lincomycin	15	0
Mez	Mezlocillin	75	28 ± 1.5
Tet	Tetracycline	30	28 ± 1
Oxc	Oxacillin	5	0
Pen	Penicillin	1 Unit	0

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CHAPTER 4

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CHAPTER FOUR

ELUCIDATING THE ROLE OF SWIMMING AND TWITCHING MOTILITY IN THE INFECTION AND COLONISATION OF ONION SEEDLINGS BY *PANTOEA ANANATIS* LMG20103

4.1 INTRODUCTION

Pantoea ananatis causes disease symptoms in a number of plant hosts, including *Eucalyptus* (Coutinho *et al*., 2002), maize (Gosczcynska *et al*., 2007; Lana *et al*., 2012) and onion (Gitaitis *et al*., 2002; Gosczcnska *et al*., 2006). Damage caused by this pathogen to agronomic crops can be severe with losses reported as high as 63% in maize (Lana *et al*., 2012) and 100% in onions (Gitaitis *et al*., 2002). The disease caused by *P. ananatis* in onions is commonly known as centre rot and was first discovered in Georgia in 1997 (Gitaitis and Gay, 1997). Symptoms include water-soaked lesions, wilting and bleaching of the leaves, and maceration of the bulbs. Diseased onions cannot be harvested as easily as healthy onions as their leaves tear away, leaving the bulb in the ground (Gitaitis *et al*., 2002).

As an emerging pathogen, *P. ananatis* is capable of proliferating in a wide variety of environments, from soil to aviation fuel tanks (Rauch *et al*., 2006) and it has recently increased both its geographical distribution and host range (Coutinho and Venter, 2009). The pathogenesis of *P. ananatis* in plants is not well understood as it lacks typical plant pathogenic factors such as the type II and III secretion systems. It does, however, have a type VI secretion system which is believed to play a vital role in its pathogenesis (de Maayer *et al*., 2011; Shyntum *et al*., 2014).

The ability of pathogenic bacteria to rapidly spread between and within host plants is largely determined by its capacity to be motile. Motility also plays a key role in initiating an infection in the host (Josenhans and Suerbaum, 2002; Soutourina and Bertin, 2003; Meng *et al*., 2011) by aiding attachment, biofilm formation (Meng *et al*., 2011; Haiko *et al*., 2013) and virulence (Haiko *et al*., 2013). While not as fast as swimming motility, twitching motility allows bacteria to rapidly colonise a surface area (Liu et al., 2001) and type IV pili are

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consequently prevalent on many bacteria (Pratt and Kolter, 1998; Liu *et al*., 2001; Cleary *et al*., 2004; Bahar *et al*., 2009). Type IV pili also play a role in attachment (Soto and Hultgren, 1999), biofilm formation (Liu *et al*., 2001; Kang *et al*., 2002), horizontal gene transfer (HGT) (Soto and Hultgren, 1999) and virulence (Bahar *et al*., 2009; Burdman *et al*., 2011).

The aims of this chapter are to investigate the role of swimming and twitching motility in the attachment and virulence of *P. ananatis* in onion seedlings. The phenotypes of the mutant strains as well as the complemented strains, generated as described in Chapter 3, will be compared to the wild-type strain both *in vitro* and *in planta*.

4.2 MATERIALS AND METHODS

The bacterial strains used in this study are listed in Table 3.1. Strains were cultured in LB broth or on LB agar at 28°C. Media was supplemented with kanamycin (50 µg/ml), ampicillin (50 µg/ml) and gentamicin (50 µg/ml) as needed.

4.2.1 Swimming motility assay

The swimming ability of the two flagellar mutants, PA(*flgK*$^-$) and PA(*motA*$^-$) and their respective complements was compared to wild-type *P. ananatis* LMG20103, in a swimming motility assay. Motility media (0.5% LB agar (1% [w/v] tryptone, 0.5% [w/v] yeast extract, 0.5% [w/v] NaCl, 0.5% [w/v] agar; pH 7.2)) (Malamud *et al*., 2011) was augmented with 0.005% triphenyltetrazolium chloride (TTC) which is colourless in its oxidised form. TTC is reduced in bacteria by a dehydrogenase enzyme to produce insoluble, red formazan crystals (MacFaddin, 1972; Thom *et al*., 1993). All bacterial strains were inoculated into the media with a sterile toothpick and incubated overnight at 28°C. The complemented strains were also included in the swimming motility assay and the assays were repeated in triplicate.

4.2.2 Twitching motility assay

The twitching motility mutants, PA(*pilA*$^-$) and PA (*pilT*$^-$), and their complements were compared to the wild-type strain in a twitching motility assay. Twitching motility assays for *R. solanacearum* and *P. aeruginosa* were effectively conducted on 1.5% media (O'Toole and Kolter, 1998; Liu *et al*., 2001) thus the media selected for the assay was 1.5% LB agar (1.5%

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[w/v] agar; pH 7.2) augmented with 0.005% TTC. Each bacterial strain was inoculated into the media with a sterile toothpick and incubated overnight at 28°C. The assays were repeated in triplicate.

To verify the twitching motility results, the wild-type strain, PA(*pilA*$^{-}$) and PA (*pilT*$^{-}$), and their complements were inoculated onto the surface of 1.5% LB agar with sterile toothpicks and incubated overnight at 28°C. The resulting colonies were viewed under a light microscope (Zeiss Axioskop 2 Plus) at 20X magnification and photographed (Zeiss Axiocam ICc3).

4.2.3 Transmission electron microscopy (TEM)

All four mutant strains as well as their complements and the wild-type strain were negatively stained with uranyl acetate and viewed under the JEOL TEM 2100F at the University of Pretoria Microscopy Unit.

4.2.4 Leaf attachment assay

Healthy onion leaves (*Allium cepa* cv. Texas Grando) from 6 week old seedlings were selected and rinsed with sterile dH_2O. The leaves were immersed in 96% ethanol to sterilise both the interior and exterior surfaces of the hollow leaf. All four mutant strains along with their complements and the wild-type strain were grown to absorbance values (O.D.) of 0.2, as determined using a Molecular Devices SpectraMax Plus 384 spectrophotometer (wavelength (λ) = 600 nm). 0.2 g of onion leaf was added to 10 ml of the bacterial culture and incubated at room temperature for 2 hours on a rotary shaker (80 rpm). The leaves were rinsed 3 times in sterile PBS (1% [w/v] NaCl, 0.025% [w/v] KCl, 0.18% [w/v] Na_2HPO_4, 0.03% [w/v] KH_2PO_4; pH 7.4) by agitating them vigorously with a vortex mixer for 15 sec to wash away any unattached bacterial cells. The leaves were ground up in 2 ml PBS and a serial dilution was plated out on LB plates amended with ampicillin to discourage the growth of other bacteria, as *P. ananatis* LMG20103 is resistant to ampicillin (Section 3.2.1). After overnight incubation the number of colonies on each plate was counted. The attachment assays were repeated in triplicate.

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4.2.5 Biofilm assay

The four mutant strains and their complements were compared to the wild-type strain in a biofilm assay. 96-well polystyrene plates were inoculated with cultures that were grown to an OD_{600} of 0.2. The plates were incubated overnight at 28°C under static conditions and rinsed six times with sterile dH_2O. Each well was stained with 180 µl of 1% crystal violet solution before a further incubation of 15 min at room temperature. The wells were rinsed again and filled with 100% ethanol. Optical density readings were taken at 600 nm. Wells containing sterile LB were used as a negative control. All biofilm assays were repeated using polyvinyl chloride (PVC) 96-well plates. The biofilm assays were repeated in triplicate to verify the results.

4.2.6 Leaf dispersal assay

All *P. ananatis* strains were grown to an OD_{600} of 0.2 and 10 µl of each strain was placed in the centre of a surface sterilised onion leaf. The inoculated leaves were maintained overnight on water agar (2% [w/v] agar; pH 7.2) at 28°C before being imprinted onto LB agar amended with 50 µg/ml kanamycin. The plates were incubated overnight at 28°C before colony growth on the plates was measured.

4.2.7 Pathogenicity trials

Healthy onion seedlings of the same age were surface sterilised with 96% ethanol and allowed to air dry. *P. ananatis* strains were grown to an OD_{600} of 0.5 and 2 µl of each strain was stab-inoculated into the leaves of two separate onion seedlings using a sterile needle and syringe. The plants were kept under normal day/night cycles for a period of 4 days before the lesions were inspected and measured. Sterile LB broth was used as a negative control. The pathogenicity trials were repeated in triplicate.

4.2.8 Statistical analysis

A single-factor ANOVA (analysis of variance) was used to calculate the variance in the results of the tests and assays performed. A single-factor ANOVA compares the hypothesis that all the samples in a data set have the same underlying probability distribution against the hypothesis that they do not all have the same underlying probability distribution.

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4.3 RESULTS AND DISCUSSION

4.3.1 Functional flagella are needed for swimming motility

Swimming motility assays were conducted using the wild-type *P. ananatis* LMG20103 and the *flgK*$^{-}$ and *motA*$^{-}$ mutants. Both the mutants were observed to have lost the ability to move through swimming motility media, whereas the wild-type strain was motile (Figure 4.1). The growth of bacteria in the media was easily discernible by the red pigmentation of the formazan and motile bacteria such as the wild-type strain, had diffused areas of growth around the point of inoculation, whereas the motility mutants only showed growth at the point of inoculation. Complementation of both the *motA*$^{-}$ and *flgK*$^{-}$ mutants was able to restore swimming motility. These results clearly indicate that *P. ananatis* needs fully functional flagella for swimming motility.

Both the *lac*-and native-promoter complemented strains were equally able to restore the wild-type phenotype. This proved to be a repeating result throughout all the tests, assays and microscopy studies conducted. As the expression of the target gene in the native-promoter complemented strains was the most similar to the wild-type strain, only the results of the native-promoter complementations are included in this chapter.

4.3.2 Twitching motility is dependent on retractable type IV pili

The type IV pilus mutants PA(*pilA*$^{-}$) and PA(*pilT*$^{-}$) had reduced twitching motility ability when compared to the wild-type strain (Figure 4.2). Complementation with functional *pilA* and *pilT* copies was able to restore twitching motility. Light microscopy showed that the edges of the twitching motility mutant colonies were smooth, whereas those of the wild-type strain and complement had irregular edges (Figure 4.3). *P. ananatis* thus needs retractable type IV pili for twitching motility.

4.3.3 Transmission electron microscopy demonstrated the absence of flagella and pili in strains PA(*flgK*$^{-}$) and PA(*pilA*$^{-}$), respectively

Flagella and pili were visible on the surface of wild-type *P. ananatis* LMG20103. Similarly, the electron micrographs of the PA(*motA*$^{-}$) and PA(*pilT*$^{-}$) mutants showed both flagella and

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type IV pili structures present in these mutants (Figures 4.4 and 4.5). By contrast, the PA(*flgK*$^-$) mutant was aflagellate (Figure 4.4), while type IV pili were absent from the PA(*pilA*$^-$) mutant (Figure 4.5). Complementation resulted in restored flagellar and pilus structures (Figures 4.4 and 4.5)

4.3.4 Swimming and twitching motility are instrumental in efficient attachment

Leaf attachment assays (Figure 4.6) were used to demonstrate the ability of the wild-type and motility mutant strains to adhere to the surface of onion leaves within a period of two hours. Wild-type *P. ananatis* LMG20103 was able to effectively attach to the leaves as plate counts averaged 89 ± 10.2 CFU/ml (average CFUs ± standard deviation). The average colony counts of PA(*flgK*$^-$) and PA(*motA*$^-$) were 10 ± 1.5 CFU/ml and 0 CFU/ml, respectively. These results indicate that flagella play a major role in attachment of *P. ananatis* to leaf surfaces. It is likely that swimming motility aids bacteria in reaching the area of attachment by not only propelling the bacteria through the natural repulsive forces that exist between the bacterial cell and the leaf surface (O'Toole and Kolter, 1998), but also by responding to chemotactic signals that allow the bacteria to locate its host and appropriate areas of attachment (Shen and Ronald, 2002; Ramos *et al*., 2004). Similar to the wild-type strain, average colony counts for PA(*flgK*$^-$)-natcomp and PA(*motA*$^-$)-natcomp were 73 ± 3.3 and 77 ± 5.1 CFU/ml CFU/ml, respectively.

Colony counts of 29 ± 1 CFU/ml and 0 were observed for the twitching motility mutants PA(*pilA*$^-$) and PA(*pilT*$^-$), respectively. Type IV pili thus also play an important role in attachment. Analysis of the *P. ananatis* genome showed that several other pili, including type I- and type III pili, are encoded on the genome (de Maayer, 2011). In the absence of the type IV pili in the PA(*pilA*$^-$) mutant, these may fulfil the attachment function (Sauer *et al*., 2000). The PA(*pilT*$^-$) mutant, however, was unable to attach. This may be due to the fact that the fully extended type IV pili were unable to retract and bring the bacterial cell into contact with the surface of the leaf (Higashi *et al*., 2007). As the type IV pili are generally longer than other pili, the extended type IV pili in the *pilT*$^-$ mutant may thus mitigate attachment of *P. ananatis* to the onion leaf by other pili (De La Fuente *et al*., 2008). The complemented strains were able to restore the wild-type phenotype as the colony counts for PA(*pilA*$^-$)-natcomp and PA(*pilT*$^-$)-natcomp were 78 ± 9.9 and 78 ± 3 CFU/ml CFU/ml, respectively.

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4.3.5 Non-motile strains form dense biofilms

All four mutant strains consistently formed biofilms that were denser than those produced by the wild type strain on both polystyrene and PVC plates. The average absorbance readings (OD_{600}) on PVC plates for the wild type strain was 0.036 ± 0.006 (average absorbance ± standard deviation), whereas those of the PA(*flgK*$^-$) and PA(*motA*$^-$) mutants were 0.113 ± 0.007 and 0.159 ± 0.021, respectively. The twitching motility mutants also showed an increased absorbance compared to the wild-type strain. PA(*pilT*$^-$) mutants formed biofilms with an average absorbance of 0.133 ± 0.004 and, although not statistically significant, the average absorbance for the PA(*pilA*$^-$) mutants was slightly higher than that of the wild-type, at 0.047 ± 0.001 (Figure 4.7). The swimming motility mutants may have formed dense biofilms as the lack of flagella prevented the cells from spreading across the wells and instead auto-aggregated into clumps. It has similarly been shown that reduced motility in *P. aeruginosa* promotes biofilm formation as the cells form 3D biofilm structures when non-motile (Klausen *et al*., 2003; Landry *et al*., 2006; Guttenplan and Kearns, 2013). Due to a decrease in motility, the cells are also less likely to disperse (Guttenplan and Kearns, 2013). Motility in *P. aeruginosa* is usually re-activated late in biofilm maturation as this gives the cells an opportunity to disperse (Guttenplan and Kearns, 2013). Similar results were also observed in *Vibrio cholerae* where flagella are essential for early biofilm formation (Pratt and Kolter, 1999; Guttenplan and Kearns, 2013). The mutant strains in this study were unable to reactivate their motility and thus no cell dispersal was able to take place. The results observed for the twitching motility mutants can similarly be explained as the loss of twitching motility would result in a localised, dense biofilm. As stated previously, type IV pili are important for biofilm formation (Liu *et al*., 2001). All complemented strains formed biofilms that were less dense than their mutant counterparts.

4.3.6 Motility allows *P. ananatis* to effectively colonise the surface of a leaf

The four mutant strains were unable to spread on onion leaves as efficiently as wild-type *P. ananatis* LMG20103. While colonies could be observed 2.1 cm ± 0.2 from the point of inoculation for the wild-type strain, PA(*flgK*$^-$), PA(*motA*$^-$), PA(*pilA*$^-$) were slightly reduced in their ability to spread across the leaf surfaces (Table 4.1; Figure 4.8). However, the spread of these mutants from the point of inoculation was not significantly different from the wild-type strain. This suggests that factors other than swimming motility and type IV pili are required

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for effective spread across the leaf surface. By contrast, the spread of the *pilT* mutant (Table 4.1) was significantly lower than that of the other three mutants and the wild-type strain. This suggests that effective twitching motility, through type IV pili retraction is essential for dispersal of *P. ananatis* across the onion leaf. The dispersion of the complemented strains on the leaf was similar to that of the wild-type strain (Table 4.1).

4.3.7 Swimming and twitching motility are essential for pathogenesis of *P. ananatis* LMG20103

Pathogenicity trials were conducted on healthy onion seedlings of the same age. Lesion length was used as a measure of virulence. Three of the four mutant strains showed reduced virulence when compared to the wild type strain (Figure 4.9; Figure 4.10). In particular, no symptoms were observed four days post inoculation (d.p.i.) on onion leaves inoculated with the *flgK*$^{-}$ and *motA*$^{-}$ mutants, indicating that swimming motility is essential for effective pathogenesis of *P. ananatis* on onion. While symptoms could be observed for the non-retractile type IV pili *pilT*$^{-}$ mutant (Table 4.2), the symptoms were far less severe than in the wild-type strain, suggesting an additive role for twitching motility towards the virulence of *P. ananatis* on onion leaves. By contrast, the unpiliated PA(*pilA*$^{-}$) mutant remained as virulent as the wild-type strain. This suggests that the type IV pili itself, does not play a major role in pathogenesis. Rather, the attachment role ascribed to the type IV pili may be complemented by the type I- and type III pili of *P. ananatis*. Similar results have been found in pathogens such as *Agrobacterium tumefaciens* (Chesnokova *et al*., 1997), *Xanthomonas axonopodis* pv. *citri* (Malamud *et al*., 2011) and *Ralstonia solanacearum* (Liu *et al*., 2001). The complemented strains were capable of initiating disease symptoms in onion seedlings and were as virulent as the wild-type strain.

4.4 CONCLUSIONS

Pantoea ananatis is a motile plant pathogen that is capable of both swimming and twitching motility. Motility is imperative in the infection cycle of most plant-pathogenic bacteria as it enables them to locate their hosts and suitable points of attachment as well as aiding in the acquisition of nutrients and dispersal (Demir *et al*., 2011; Ichinose *et al*., 2013). Similarly, roles in surface adhesion, twitching motility, avoidance of host defence systems, biofilm

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formation and pathogenicity have been ascribed to type IV pili in the plant pathogens *Acidovorax avenae, R. solanacearum* and *Pseudomonas syringae* (Kang *et al*., 2002; Bahar *et al*., 2009; Burdman *et al*., 2011). Here, by means of knock-out mutagenesis and phenotypic assays, we illustrate that both flagellar motility and type IV piliation play a role in the infection process of *P. ananatis* on onions.

Knock-out mutagenesis of the *P. ananatis* LMG20103 *flgK* gene, which codes for a flagellar hook-associated protein, resulted in non-flagellated cells, while the flagellar motor protein-coding *motA*$^-$ mutant was flagellated, but non-motile. As has been observed with the well-characterized phytopathogens *R. solanacearum* and *Pseudomonas aeruginosa* (O'Toole and Kolter, 1998; Kang *et al*., 2002; Meng *et al*., 2011), both the aflagellate (*flgK*$^-$) and motility-disrupted (*motA*$^-$) mutants were incapable of swimming motility and were reduced in their ability to attach to onion leaves. Infection assays also showed that both of these mutants were non-pathogenic. The non-pathogenesis of the swimming motility mutants may thus be correlated to their inability to attach to the host plant or move to the site of infection. Alternatively, other pathogenicity-related functions have been ascribed to the flagella. For example, virulence-associated proteins have been known to be secreted through the flagellar filament in *Yersinia enterolitica* and *Campylobacter jejuni* (Kirov, 2003, Fernando *et al*., 2007).

In addition to type IV pili, the genome of *P. ananatis* LMG20103 codes for several additional putative fimbriae and pili including type I- and type III pili that are known to play a role in attachment in other phytopathogens (Haahtela *et al*., 1985; Korhonen *et al*., 1987; Sauer *et al*., 2000; de Maayer, 2011). The loss of type IV pili in the *pilA*$^-$ mutant reduced its ability to attach to onion leaves. As limited attachment was still able to occur, it is likely that the shorter type I and type III pili were involved in the attachment of the mutant to the host. By contrast, no attachment to onion leaves was observed for the PA(*pilT*$^-$) mutant. This can be ascribed to the role of type IV pili in the initial "loose attachment" phase. Cells loosely tethered to the surface by type IV pili will descend towards the surface, where an intimate adherence can be initiated (Higashi et al. 2007; Sun et al. 2000). The inability of the *pilT*$^-$ mutant to retract may thus result in the detachment of the bacterial cells during the more

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rigorous washing steps employed in the attachment assays. Leaf dispersion was likewise significantly reduced in the *pilT*$^{-}$ mutant, suggesting that twitching motility, rather than swimming motility is required for the spread of *P. ananatis* across the leaf surface. Alternatively, as the *pilT* gene is required for retraction of the type IV pili, the non-retractile type IV pili filament in this mutant may have interfered with the functioning of the cell's peritrichous flagella and resulted in increased drag of the cell.

The PA(*pilA*$^{-}$) mutant was as virulent as the wild-type strain suggesting that, unlike has been observed in many other phytopathogenic bacteria, the type IV pili of *P. ananatis* are not required for pathogenesis, although they do play a role in initiating the infection process. This may be due to the complementary role of the type I- and type III pili in the attachment process. Due to the loss of attachment, the *pilT*$^{-}$ mutants showed reduced pathogenicity on onions. The type IV pili mutants also showed reduced dispersal on onion leaves. Both the flagellar and type IV pili mutants formed a denser biofilm than the wild-type strain. The cells auto-aggregated and formed clumps and the loss of motility of the bacteria may have resulted in their inability to spread across the well, thus forming a dense biofilm.

Our results provide preliminary insights into how *P. ananatis* may colonise its host plant and specifically demonstrates the role of motility in attachment, biofilm formation, leaf dispersal and pathogenicity. Flagellar motility affects both attachment to the leaf surfaces, biofilm formation and has a marked effect on symptom development on onion leaves, while the type IV pili and twitching motility are required for initial attachment and leaf dispersal, but do not have a direct effect on the virulence of *P. ananatis*. Taken in combination, our results indicate that both flagellar and twitching motility play an important role in the infection cycle of *P. ananatis* on onion leaves.

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FIGURES

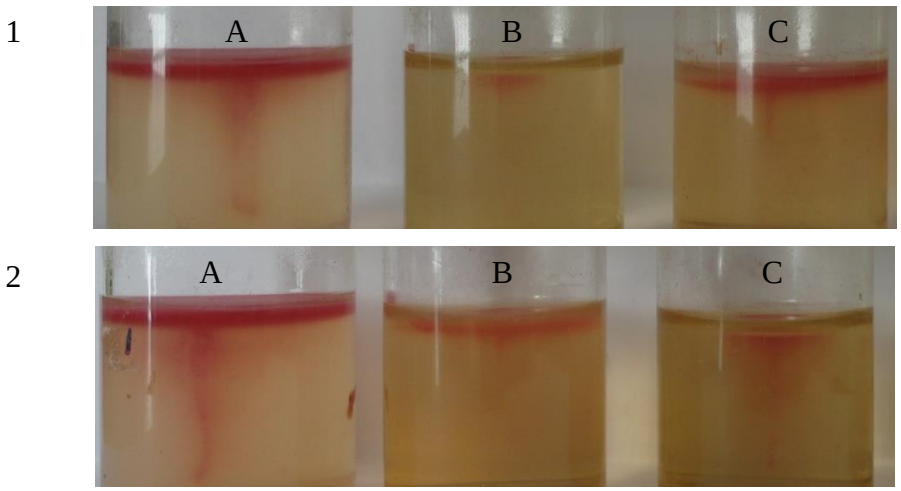

Figure 4.1: Swimming motility assay. Motility could be observed in the wild-type *P. ananatis* LMG20103 (1A and 2A), as well as the complemented strains (1C and 2C), while reduced swimming motility was observed for the PA(*flgK*$^{-}$) (1B) and PA(*motA*$^{-}$) (2B) mutants.

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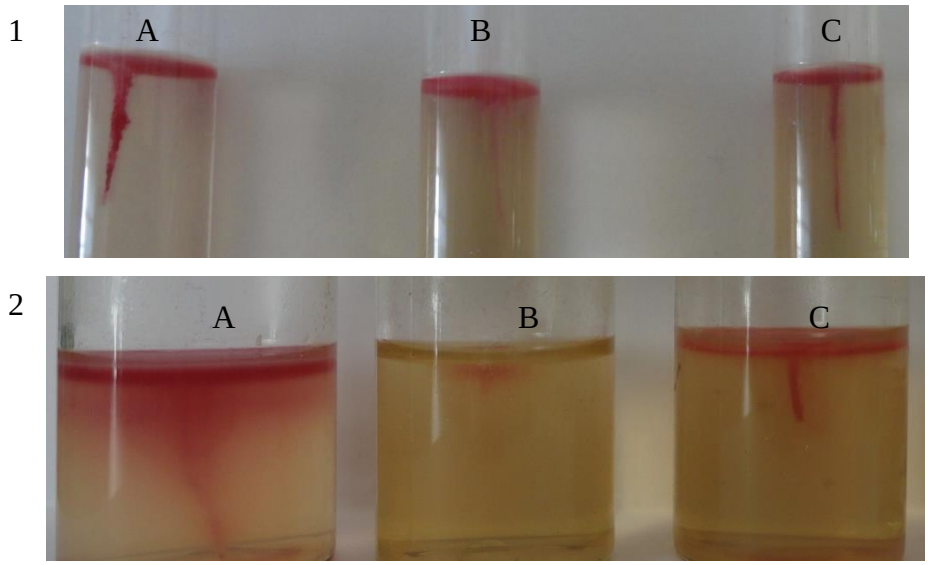

Figure 4.2: Twitching motility assay. Twitching motility could be observed in the wild-type *P. ananatis* LMG20103 (1A and 2A), as well as the complemented strains (1C and 2C), while reduced twitching motility was observed for the PA(*pilA*$^{-}$) (1B) and PA(*pilT*$^{-}$) (2B) mutants.

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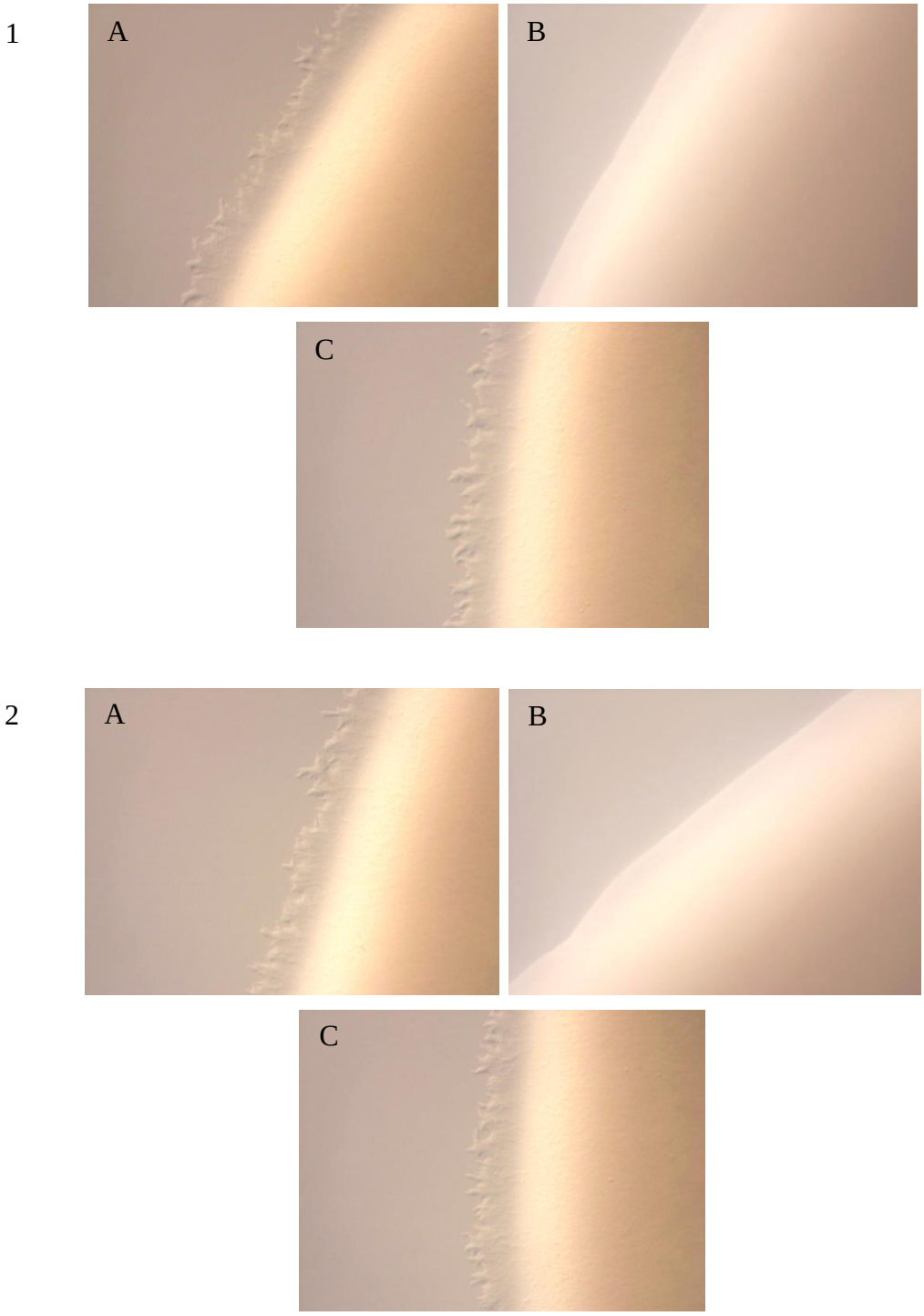

Figure 4.3: Light microscopy of the twitching motility mutants showing the smooth edges of the PA(*pilA*$^{-}$) (1B) and PA(*pilT*$^{-}$) mutant colonies (2B) compared to rough edges observed for colonies of the wild-type (A) and complemented strains (C). Colonies were viewed at a 20X magnification.

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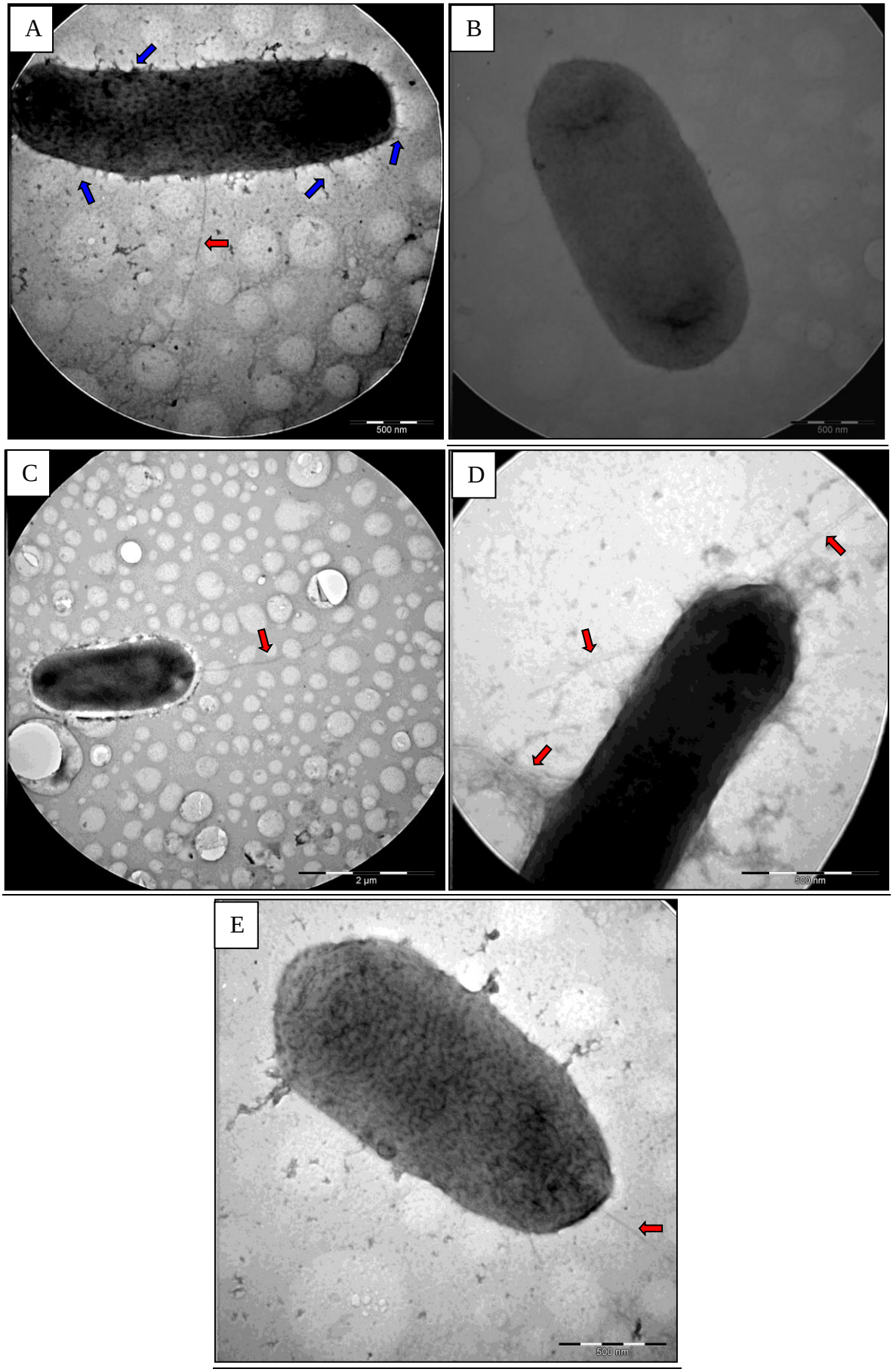

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Figure 4.4: Transmission electron microscopy of the swimming motility mutants. Wild-type *P. ananatis* LMG20103 (A) displayed both flagella (red arrows) and pili (blue arrows) whereas PA(*flgK*$^{-}$) (B) was aflagellate. The PA(*motA*$^{-}$) mutant (C) and both complemented strains (D – E) had visible flagella on their surfaces.

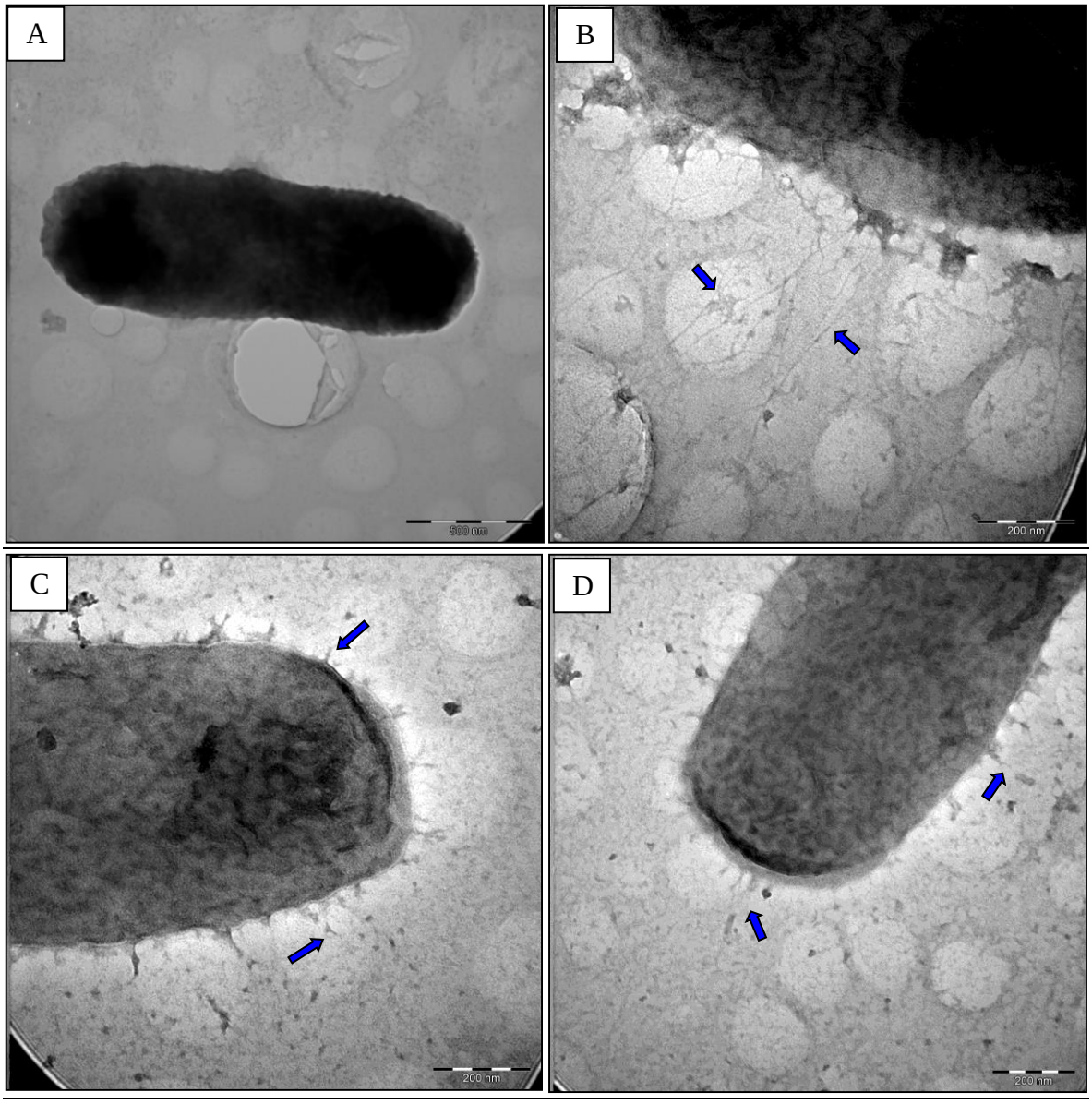

Figure 4.5 Transmission electron microscopy of the twitching motility mutants. There were no discernible pili on PA(*pilA*$^{-}$) (A) whilst it was observed that the PA(*pilT*$^{-}$) mutant (B) and both complements (C - D) were piliated.

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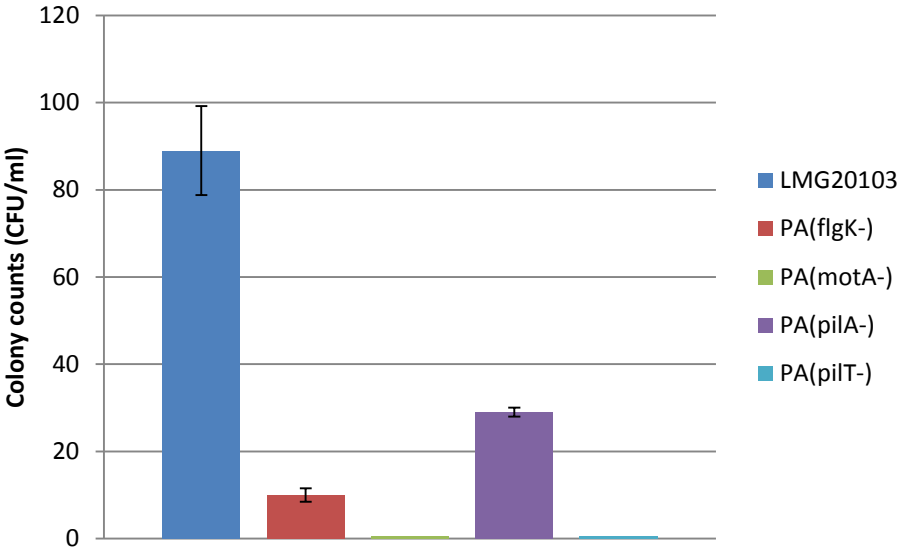

Figure 4.6: Leaf attachment assay. All four mutant strains showed significant reduction in the ability to attach to onion leaves. The error bars denote the variability of the results when combining the data from all the replicates.

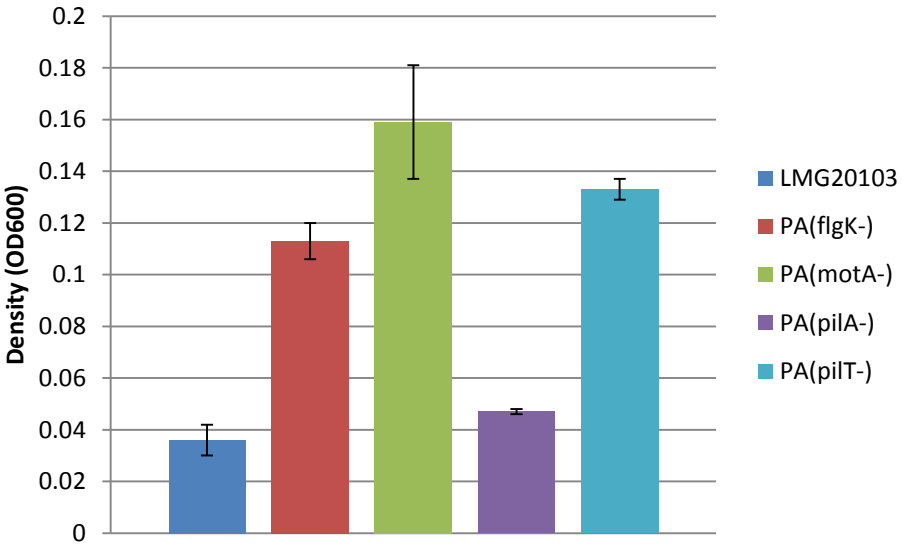

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Figure 4.7: Biofilm assay. The absorbances were measured using a spectrophotometer (wavelength = 600 nm). The PA(*flgK*$^-$), PA(*motA*$^-$), PA(*pilA*$^-$) and PA(*pilT*$^-$) mutants formed denser biofilms than wild-type *P. ananatis* LMG20103. The results are displayed as the mean ± standard deviation of the replicates.

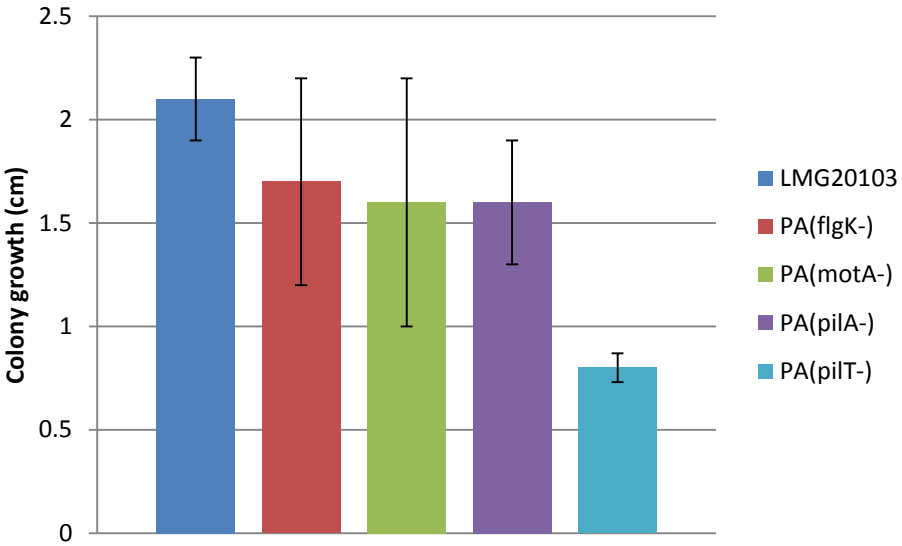

Figure 4.8: Leaf dispersion assay. Whilst all the mutant strains were reduced in their ability to spread across the surface of a leaf, PA(*pilT*$^-$) was significantly reduced and demonstrates the need for retractile type IV pili in successful leaf surface colonisation. The error bars are representative of the standard deviation of the mean.

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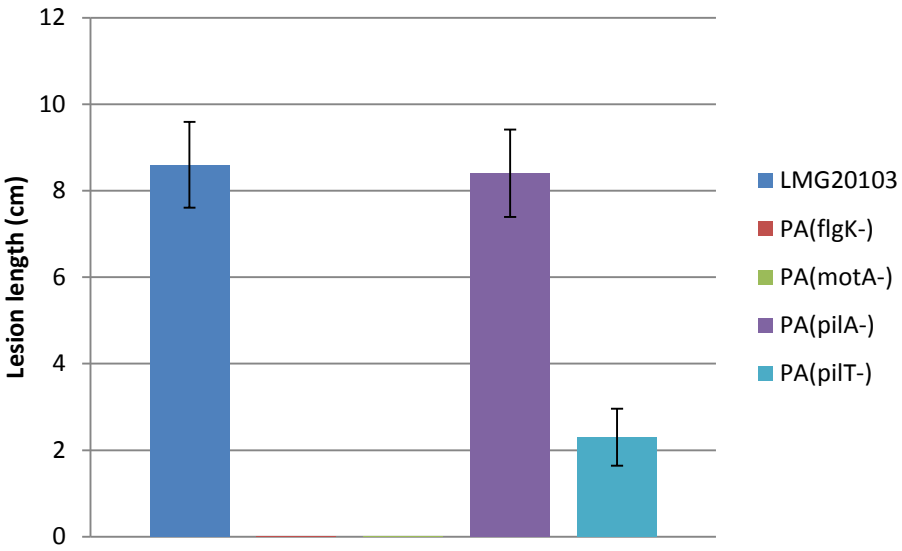

Figure 4.9: Pathogenicity trials on onion seedlings. Both the flagellar mutants were unable to form lesions on onions whereas the PA(*pilA*$^{-}$) mutant was as virulent as the wild-type strain. Onion plants inoculated with PA(*pilT*$^{-}$) displayed disease symptoms, but they were not as severe as those seen in *P. ananatis* LMG20103 four d.p.i. The error bars denote the standard deviation of the mean.

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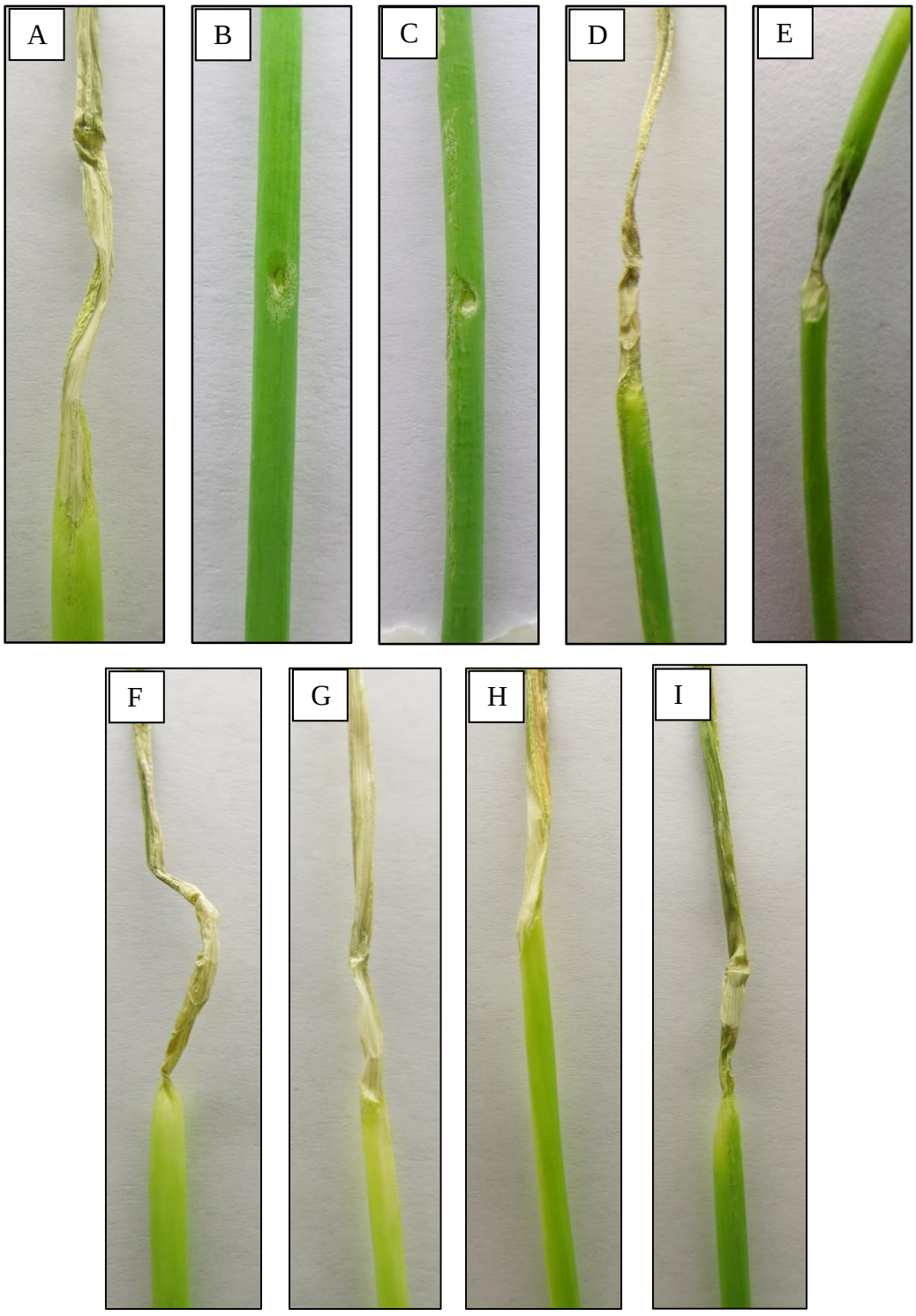

Figure 4.10: Photos of the pathogenicity trials. Both *P. ananatis* LMG20103 (A) and PA(*pilA*$^{-}$) (D) caused lesions from the point of inoculation to the tip of the leaf whereas PA(*flgK*$^{-}$) (B) and PA(*motA*$^{-}$) (C) did not display any symptoms. PA(*pilT*$^{-}$) formed lesions that were not as severe as either the wild-type or PA(*flgK*$^{-}$). The complemented strains (F-I) were able to form lesions on onion seedlings.

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TABLES

Table 4.1: Dispersion of *P. ananatis* strains across the surface of an onion leaf from the point of inoculation

***P. ananatis* strain**	**cm ± standard deviation**
LMG20103	2.1 ± 0.2
Pa(*flgK*$^-$)	1.7 ± 0.5
Pa(*motA*$^-$)	1.6 ± 0.6
Pa(*pilA*$^-$)	1.6 ± 0.3
Pa(*pilT*$^-$)	0.8 ± 0.07
PA(*flgK*$^-$)-natcomp	2.0 ± 0.01
PA(*motA*$^-$)-natcomp	2.1 ± 0.2
PA(*pilA*$^-$)-natcomp	1.9 ± 0.08
PA(*pilT*$^-$)-natcomp	1.8 ± 0.03

Table 4.2: Average lesion lengths caused by *P. ananatis* strains on onion

***P. ananatis* strain**	**cm ± standard deviation**
LMG20103	8.6 ± 0.99
Pa(*flgK*$^-$)	0
Pa(*motA*$^-$)	0
Pa(*pilA*$^-$)	8.4 ± 1.01
Pa(*pilT*$^-$)	2.3 ± 0.66
PA(*flgK*$^-$)-natcomp	6.8 ± 0.35
PA(*motA*$^-$)-natcomp	7.8 ± 0.44
PA(*pilA*$^-$)-natcomp	7.8 ± 1.28
PA(*pilT*$^-$)-natcomp	8.1 ± 0.36

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CHAPTER 5

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CHAPTER FIVE

DRAFT GENOME SEQUENCES OF THE ONION CENTRE ROT PATHOGEN *PANTOEA ANANATIS* PA4 AND MAIZE BROWN STALK ROT PATHOGEN *P. ANANATIS* BD442

5.1 INTRODUCTION

Pantoea ananatis is found in diverse natural environments and causes disease symptoms in a broad range of host plant species (Coutinho and Venter, 2009) including maize (Paccola-Meirelles *et al*., 2001; Goszczynska *et al*., 2007), rice (Yan *et al*., 2010), and other economically important agricultural crops. *P. ananatis* was first isolated in South Africa from *Eucalyptus* seedlings displaying blight and dieback symptoms (Coutinho *et al*., 2002). Since then it has been isolated as the causative agent of brown stalk rot of maize in South Africa (Goszczynska *et al*., 2007). It was also isolated from onion seeds and has been linked to centre rot of this host (Goszczynska *et al*., 2006). Here we report the draft genome sequences of two virulent *P. ananatis* strains isolated from maize (BD442) and onion seed (PA4) in South Africa. These strains were obtained from the Plant Pathogenic and Plant Protecting Bacterial Culture Collection, Agricultural Research Council – Plant Protection Institute, South Africa.

Weller-Stuart, T., Chan, W. Y., Coutinho, T. A., Venter, S. N., Smits, T. H. M., Duffy, B., Goszczynska, T., Cowan, D. A., de Maayer, P., 2014. Draft genome sequences of the onion center rot pathogen *P. ananatis* PA4 and maize brown stalk rot pathogen *P. ananatis* BD442. *Genome Announcements* **2** (4): e00750-14. doi:10.1128/genomeA.00750-14

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5.2 MATERIALS AND METHODS

The genomes of *P. ananatis* BD442 and PA4 were sequenced using the Illumina HiSeq 2500 platform (2x 51 bp shotgun sequencing). This yielded 63,960,136 (BD442) and 72,985,976 (PA4) paired-end reads representing an estimated coverage of 652x (BD442) and 744x (PA4), respectively. The genomes were assembled *de novo* using the Velvet short-read assembler plugin (Zerbino and Birney, 2008) of the Geneious Server (Biomatters Ltd., Auckland, New Zealand) with approximately 16,000,000 reads per strain. Further gap closure was done by scaffolding the genomes against the complete *P. ananatis* clinical strain LMG5342 (De Maayer *et al*., 2012) and *Eucalyptus* strain LMG20103 (De Maayer *et al*., 2010) using Mauve version 2.3.1 (Darling *et al*., 2004).

5.3 RESULTS AND DISCUSSION

The *P. ananatis* BD442 genome was assembled into eleven contigs, with a total size of 4.80 Mb, a mean G+C content of 53.59 % and average contig length of ~436 kb, while that of PA4 was assembled into seventeen contigs, with a genome size of 5.16 Mb, a mean G+C content of 53.56% and average contig length of ~303 kb. Both assemblies incorporate complete circular plasmids, pPANA1BD442 (~353 kb; G+C% = 51.13%) and pPANA1PA4 (~313 kb; G+C% = 52.17%), that belong to the Large *Pantoea* plasmid-1 group, which plays a major role in the evolutionary diversification of *Pantoea* spp. (De Maayer *et al*., 2012). The genomes were annotated using the Rapid Annotations using Subsystems Technology (RAST) server (Aziz *et al*., 2008). The genomes code for 4,673 (BD442) and 5,111 (PA4) proteins, respectively. Of these, 3,749 proteins are conserved between the two strains, while variability can largely be ascribed to prophage integration (De Maayer *et al*., 2014).We previously described three Type VI secretion system (T6SS) loci in *P. ananatis* that play a role in animal and plant pathogenesis (De Maayer *et al*., 2011). All three loci (T6SS-1,-2 and -3) are present in *P. ananatis* PA4 whereas T6SS-3 is missing in BD442 (De Maayer *et al*., 2014; De Maayer *et al*., 2011). These genomes will provide new insights into the pathogenic lifestyle of *Pantoea ananatis* and how it is able to cause disease symptoms on such a broad range of host plants.

Nucleotide accession numbers. These Whole Genome Shotgun projects have been deposited in DDBJ/ENA/GenBank under the accession numbers JMJK00000000 (*P. ananatis* PA4) and

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JMJL00000000 (*P. ananatis* BD442). The versions described in this paper are the first version, JMJK01000000 (PA4) and JJML01000000 (BD442).

5.5 ACKNOWLEDGEMENTS

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SUMMARY

Pantoea ananatis is a ubiquitous bacterium capable of infecting an extensive number of plants species. In South Africa specifically it infects *Eucalyptus*, onion and maize and disease outbreaks result in substantial economic losses each year. *P. ananatis* is capable of both swimming and twitching motility which potentially play a role in pathogenicity as many other well-known phytopathogens rely on motility to locate and infect their hosts.

Genome comparisons between four fully sequenced and annotated *P. ananatis* strains (AJ13355, LMG20103, PA13 and LMG5342) as well as comparisons to five closely related enterobacterial strains (*P. vagans* C9-1, *Pantoea* sp. At-9b, *Erwinia amylovora* CFBP1430, *Salmonella enterica* serovar Typhimurium LT2 and *Eschericia coli* K-12 MG1655) were performed. This revealed that both the flagellum and type IV pilus biosynthetic genes are well-conserved, both in sequence and gene organization, among the compared *P. ananatis* strains and the enterobacterial comparators. Phylogenetic analyses suggest that both flagellar motility and type IV pilus-mediated twitching motility are ancestral traits, which have been vertically maintained through the speciation events among the *Enterobacteriaceae*. One notable anomaly is the presence of multiple copies of the *fliC* gene on the genomes of the different *P. ananatis* strains, and these may play a role in phase variation. As flagellin is a highly antigenic protein, phase-variable expression of distinct *fliC* genes could allow *P. ananatis* to remain undetected within its host. A non-conserved region coding for proteins that play a role in flagellin glycosylation was observed in the *P. ananatis* strains. The type IV pili, which is one of eight putative pili encoded on the *P. ananatis* genome, are very well conserved amongst the compared *P. ananatis* strains. The genomes of two additional *P. ananatis* strains, namely PA4 and BD442, were released during the course of this research. The mining of these genomes will provide new insights into the pathogenic lifestyle of *P. ananatis*.

Four motility mutants of *P. ananatis* LMG20103 were generated using genetic recombineering techniques. To elucidate the role of swimming motility in the colonisation and pathogenicity of *P. ananatis* on onions, the two flagellar genes *flgK* (aflagellate and non-motile) and *motA* (flagellated and non-motile) were disrupted with a kanamycin resistance gene. Two type IV pilin genes, *pilA* (unpiliated) and *pilT* (piliated but unable to retract), were similarly disrupted. The mutations were confirmed and subsequently complemented. By

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comparing the mutants and complemented strains to the wild-type strain, it was found that swimming motility significantly affected the ability of *P. ananatis* to attach to onion leaves and the swimming motility mutants were non-pathogenic. While it is commonly accepted that type IV play a pivotal role in attachment of plant pathogenic bacteria to their host tissues, the lack of type IV pili in *P. ananatis* mutant strains may, potentially, be complemented by the presence of other pili, such as type I and type III pili. Type IV pili play a role in initiating the infection process as they allow the bacterial cell to form an intimate attachment with the host surface. Both of the flagellar and both of the type IV pilus mutants also formed denser biofilms than the wild-type strain as the cells auto-aggregated and were unable to spread or disperse. While the *flgK*$^{-}$, *motA*$^{-}$ and *pilA*$^{-}$ mutants were unable to spread across a leaf surface as effectively as the wild-type strain, dispersion of the *pilT*$^{-}$ was significantly reduced which suggests that twitching motility is instrumental in the spread of *P. ananatis* across the surface of its host. These results demonstrated how *P. ananatis* uses both swimming and twitching motility as a tool to attach to, colonise and infect its host.

Currently no effective management practices for the control of the phytopathogen *P. ananatis* and the disease symptoms it causes on a broad range of host plants are in place. The results generated in this study provide a clearer understanding of the colonisation and pathogenicity of *P. ananatis.* These may eventually contribute to the development of more effective strategies for controlling this pathogen.

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