Proteomic and functional characterisation of biofilm formation in *Xylophilus ampelinus*

By

Nompumelelo Philile Praiseworth Nyembe

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Supervisor: Prof. N. N. Ludidi

Supervisor: Dr. Y. Petersen

Co-supervisor: Prof. B. K. Ndimba

ABSTRACT

Xylophilus ampelinus, the causal agent of bacterial blight of grapevine, inhabits the vascular tissues of infected plants where the cells appear to form assemblages resembling biofilms. Bacterial blight of grapevine affects vine-growing areas in Europe and parts of the Mediterranean, Japan, and South Africa. Very little is known about the genetics and pathogenicity factors of the bacterium. The aim of this study was to characterize the biofilm formation process of *X. ampelinus* through the analysis of its biofilm proteome and functional characterization of the type IV pili (T4P). Biofilm formation allows the bacteria to grow on surfaces, produce and respond to signals, and alter their phenotypes through gene expression to promote adaptation and virulence. To characterize the biofilms formed by *X. ampelinus*, an *in vitro* biofilm formation assay was used to identify the stages of biofilm formation. The four major stages of biofilm formation identified, namely attachment, initial biofilm (micro-colony) formation, biofilm maturation, and dispersal, occurred at day three, five-seven, ten, and fifteen, respectively. When comparing the protein profiles from the initial and mature biofilm stages against the planktonic culture protein profile, 254 spots - showing a two-fold change - were considered to be differentially expressed. Out of 99 differentially expressed proteins selected for identification, fifty-nine protein spots yielded 82 protein identities that were assigned to seven functional categories including cellular processes, environmental adaptation, environmental information processing, genetic information processing, metabolism, membrane transport, and proteins of unknown function. Most proteins involved in genetic information processing were induced in abundance during both stages of biofilm formation whereas most proteins involved in carbohydrate, amino acid, and energy metabolism were reduced. Among the proteins identified, a PAS domain S-box-containing protein/diguanylate cyclase (GGDEF)-like protein was induced during the initial biofilm stage, while an indication that *X. ampelinus* biofilm formation process and regulation require the secondary messenger cyclic di-GMP, this was further confirmed by detection of proteins involved in two of the c-di-GMP-targeted pathways required for cellulose and poly-N-acetylglucosamine (PNAG) biosynthesis. Bioinformatic analysis also identified five proteins with similarity to the proteins encoded in the *rpf* (regulation of pathogenicity factors) cluster of phytobacteria, which are required for DSF quorum-sensing signal synthesis and perception. Furthermore, the role of T4P in attachment and biofilm formation was identified through the functional characterization of six *X. ampelinus* genes, namely *pilA*, *pilB*,

pilC, *pilD*, *pilQ*, and *pilU*. Scanning transmission electron microscopy (STEM) showed that *pilA*, *pilB*, *pilC*, *pilD*, and *pilQ* genes are indispensable in the biogenesis and expression of T4P, while the twitching motility gene, *pilU*, was not required for T4P expression. All T4P mutants showed defects in the ability to twitch on agar surface under inducing conditions. Consequently, T4P mutants (*pilA*, *pilB*, *pilC*, and *pilD*) were impaired in their ability to form biofilms *in vitro* while *pilQ* and *pilU* mutants were not affected. *In planta* studies of the mutant and complemented T4P strains showed that *pilA*, *pilB*, *pilC*, *pilD*, and *pilQ* deficient mutant strains were unable to form biofilms both at the inoculation point (IP) and at distal parts from the IP, indicating that T4P are required for host-surface colonization and establishment of bacteria inside the host plant. The *pilU* mutant retained the wild-type phenotype in biofilm formation both *in vitro* and at the point of inoculation *in planta*, however, the same mutant failed to form micro-colonies and biofilms at distal parts from the IP indicating that the translocation of the cells across surfaces requires the retraction function of the T4P that drives twitching motility. The observed role of T4P in biofilm development inside plants and the reduced virulence exhibited by all the T4P mutants indicate that T4P is required for *in planta* biofilm formation and surface colonization through twitching motility and that T4P is required for the virulence of *X. ampelinus* in its host, grapevine. Together, the biofilm proteome analysis and functional characterization of *X. ampelinus* T4P provide new insights into biofilm formation, its regulation and contribution to the virulence of *X. ampelinus*.

KEY WORDS

Xylophilus ampelinus

Vitis vinifera

Bacterial blight

Pathogenicity factors

Biofilm formation

Biofilm regulation

Initial biofilms

Mature biofilms

Type IV pili (T4P)

Twitching motility

Virulence

DECLARATION

I, Nompumelelo Phililе Praiseworth Nyembe, declare that the thesis, which I hereby submit for the degree philosophiae doctor at the University of the Western Cape, 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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Nompumelelo Phililе Praiseworth Nyembe

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Date

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LIST OF ABBREVIATIONS

ACN	Acetonitrile
ANOVA)	Analysis of variance
bp	Base pair
BLAST	Basic Local Alignment Search Tool
BLASTp	Protein-protein BLAST
BSA	Bovine serum albumin
c-di-GMP	Cyclic di-guanosine monophosphate
CBB	Coomassie brilliant blue
cds	Coding sequence
cfu	Colony-forming unit
cm	Centimeter
CRP	CAMP receptor protein
CTAB	Cetyl Trimethyl Ammonium Bromide
1D	One dimension
2D	Two dimension
3D	Three dimension
Da	Dalton
DNA	Deoxy-ribonucleic acid
dNTPs	Deoxynucleotide triphosphates
DSF	Diffusible signal factor
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EPS	Extracellular polymeric substances
GLM	Generalized linear model
IP	Inoculation point
IPG	Immobilized pH gradient
kDa	Kilo Dalton
kV	Kilo Volts
LB	Luria-Bertani
M	Molar
MALDI ToF	Matrix-assisted laser desorption/ionization time-of-flight
mg	Milligram
ml	Millilitre
mm	Millimeter
mM	Micro-molar
MOWSE	Molecular Weight Search
MS	Mass spectrophotometry
MW	Molecular weight

NCBI	National Center for Biotechnology Information
nm	Nano meter
nPCR	Nested polymerase chain reaction
ORF(s)	Open reading frame(s)
PAS	Per-ARNT-Sim
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pI	Isoelectric point
PMSF	Phenylmethylsulfonyl fluoride
PVPP	Polyvinylpyrrolidone
rDNA	Recombinant DNA
rpf	Regulator for pathogenicity factors
rpm	Revolutions per minute
QS	Quorum sensing
SAS	Statistical Analysis Software
SDS	Sodium duodecyl sulphate
SDS-PAGE	Sodium duodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
SMART	Simple Modular Architecture Research Tool
SOE	Synthesis by Overlap Extension
STEM	Scanning transmission electron microscope
T4P	Type IV pili
T4SS	Type IV secretion system
TBE	Tris Borate EDTA
tBLASTn	Protein sequence and translated DNA database
TCA	Trichloroacetic acid
TEM	Transmission electron microscope
TEMED	N,N,N',N'-Tetramethylethylenediamine
TFA	Trifluoroacetic acid
UV	Ultraviolet
V	Volts
Vhrs	Volt hours
wt	Wild-type
YPG	Yeast peptone glucose
YPGA	Yeast peptone glucose agar

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1. CHAPTER 1: Literature Review

1.1. *Xylophilus ampelinus*

Xylophilus ampelinus, is a phytobacterium causing bacterial blight disease of grapevines (Panagopoulos 1969). To date, grapevine is the only known host of *Xylophilus ampelinus* (EPPO Bulletin 2009). Bacterial blight of grapevines is a chronic and destructive disease previously confined to vine-growing areas in Europe, Mediterranean as well as South Africa. Most recently, the *X. ampelinus* was identified in Japan, following the first disease outbreak in Hokkaido (Komatsu and Kondo 2015). In France, the disease symptoms were first observed in the vineyards of Oléron Island and the disease was known as 'maladie d'Oléron' and later named 'necrose bactérienne' (Prunier et al. 1970). In Spain and Italy, the disease was named 'necrosis bacteriana' and 'mal nero', respectively (Alvarez 1997; Grasso et al. 1979). The same disease was called 'tsilik marasi' in Greece and 'vlamsiekte' in South Africa, the latter translating to 'flaming disease' (Erasmus et al. 1974; Matthee et al. 1970; Panagopoulos 1969). Although absent in many countries, the disease is still listed as present in Moldova, France, Greece, Italy, Slovenia, Spain, Japan, and South Africa.

1.1.1. Taxonomy, Characteristics, and Detection

Bacterial blight of grapevine was originally described in Greece (Crete) and the pathogen was named *Xanthomonas ampelina* (Panagopoulos, 1969), which was later transferred to the new genus *Xylophilus* based on DNA and RNA hybridization studies (Willems et al., 1987). *Xylophilus ampelinus* belongs to the family Comamonadaceae, a beta-subclass of proteobacteria (Willems et al. 1992). The bacterium is a Gram-negative aerobe, non-spore forming, with a single polar flagellum, which produces a yellow pigment, and metabolizes carbohydrates oxidatively. In addition to these characteristics, the bacterium is also catalase positive, oxidase negative, and grows on L-glutamine but not calcium lactate (Serfontein et al. 1997). Its genome has a predicted G+C content of 68% (Panagopoulos 1969). *Xylophilus ampelinus* grows on nutrient agar (NA) and yeast-extract-peptone-glucose-agar (YPGA) media (Erasmus et al. 1974; Manceau et al. 2000). The growth is slow and optimal at 26 °C on NA and 28 °C on YPGA media (Dreo et al. 2005;

Panagopoulos 1987; Willems et al. 1991). When grown under these conditions, slight yellow-pigmented colonies start to appear between five and ten days post-culturing (Willems et al. 1992).

Early detection methods developed for *X. ampelinus* include biochemical and serological tests (Erasmus et al. 1974), while general confirmation tests may include a Gram reaction, catalase, and oxidase test, urease production and lypolization of Tween 80 (Serfontein et al. 1997). At a later stage, molecular detection methods including nested PCR and real-time PCR assays were established for the detection of *X. ampelinus* in grapevine plants (Botha et al. 2001; Dreo et al. 2007).

1.1.2. Disease Transmission, Symptoms, and Management

Xylophilus ampelinus enters the plant through natural and artificial wounds or openings and attacks the vascular system of the vines (Bradbury 1991; Grall and Manceau, 2003). The pathogen is readily transmitted from one plant to another with pruning tools (Ridé et al. 1977) where it enters healthy tissues mainly through pruning wounds, especially in wet and windy weather. The bacteria then spread to other shoots in the early summer because of warm, moist conditions, and the disease spread is favoured by overhead sprinkler irrigation. The bacterium is able to survive in the wood, and may be transmitted from nursery to nursery in infected cuttings. The primary inoculum can be latently present in plant material such as rootstocks, scions, dormant buds, and bud chips. In affected vineyards, *X. ampelinus* overwinters within the vascular tissues of woody cankers present on trunks, branches and shoots, leaves, and within the bleeding sap (Manceau et al. 2005).

Initial studies by Grall and Manceau (2003), reported three types of inoculation methods which were aimed at understanding the mode of transmission and colonization trends associated with this bacterium. From this study, it was observed that epiphytic colonization following artificial spraying of plants with bacterial inoculum is important for disease development. It was also observed that when inoculated, the woody cuttings do not support the colonization of emerging plantlets, however, the bacteria accumulate in xylem vessels of such cuttings proving its importance for multiplication and conservation of bacteria rather than a route for plant colonization. The final observation of bacterial progression in the stems following wound inoculations, showed that the bacteria can progress to the crown through the xylem vessels where

it appeared to form biofilms. The later reports showed that under natural conditions, the bacteria may not undergo epiphytic phase, but may be transmitted through the bleeding sap (Grall et al 2005). In this study, the bleeding sap leaking from pruning wounds facilitated both primary and secondary inoculations (Grall et al. 2005). Interestingly, a recent study reported that *X. ampelinus* was only detected in buds, but not in the sap and vascular bundles (Komatsu and Kondo 2015).

The most characteristic symptom of bacterial blight is the formation of cracks and cankers on spurs, branches, young shoots, petioles, and peduncles (Panagopoulos 1987). More disease symptoms can be observed on leaves, and bunches. Figure 1.1 shows some of the disease symptoms that could be observed on shoots, bunches, and leaves. On shoots, symptoms are observed in early spring at the lower two to three nodes of the shoots. Initially, linear reddish-brown streaks appear and extend from the base to the shoot tip (Figure 1.1D). The cankers later extend slowly upwards on the growing shoots (Figure 1.1A). Generally, the affected buds may not sprout, while the shoots may become weakened and in most cases become dry. In severe cases, the extended cankers grow several centimetres long. The branches may also weaken, becoming prone to breaking. Symptoms on leaves usually appear as reddish or dark spots sometimes with an oily appearance, surrounded by a yellowish halo. Marginal drying of leaves may also occur, discolouring a major part of the leaf leading to detachment (Figure 1.1B and C). The flowers also may acquire a reddish coloration and harden in consistency.

Figure 1.1. Bacterial blight symptoms on susceptible *Vitis vinifera* cultivars. A) Necrotic lesions on young shoots, B) "flaming" discolouration of leaf margins, C) withering of bunches and leaves, and D) necrotic cracks and cankers along the shoot. The photographs are used with the permission of W. E. Langenhoven (ARC Infruitec-Nietvoorbij).

Due to the lack of effective control measures, bacterial blight disease of grapevines, caused by *X. ampelinus* continues to be a problem within the table grape industry in the Western Cape province of South Africa. Throughout the past years, the management of the disease has been through viticultural practices such as destroying infected shoots, carrying out pruning late in dry seasons and disinfecting pruning tools thoroughly before the pruning process. However, these efforts do not prevent the disease occurrence, but only reduce the spread of the disease and are ineffective in latent infections where early detection is not possible.

1.1.3. Molecular Characterisitics of *X. ampelinus*

Recently, the genome sequence of the type strain *Xylophilus ampelinus* CECT 7646 (LMG 5856) was made available on NCBI. The genome of this strain (GenBank assembly accession: GCA_003217575.1) is 3,731,505 bp long with 3360 coding domains and 3143 protein sequences. The availability of *X. ampelinus* genome sequence will help the current and future studies aimed at understanding the growth, development and pathogenicity of this bacterium. In 2014, a proteomic study of *X. ampelinus* identified some of the important factors associated with the virulence of the pathogen, such as ATP-dependent proteases, two peptidyl-prolyl isomerases, and diguanylate cyclase among others (Sevillano 2014). Although it was not mentioned in this study, diguanylate cyclases participate in the formation of the ubiquitous second messenger, cyclic-di-GMP, involved in bacterial biofilm formation and persistence (Römling et al. 2013; Ryan et al. 2007). The role of c-di-GMP in the regulation of biofilm formation is discussed further in section 1.2.2 and 1.2.2.2. In addition, a molecular study using transposon mutagenesis identified the *mntH* gene which was characterized as a virulence factor (Sevillano et al. 2014). To date, no additional pathogenicity factors have been identified in this pathogen.

1.2. Biofilm Formation

Biofilm formation is a complex process required by most bacterial species to complete their cell cycle in the environment. A biofilm is defined as a well-structured community of bacterial cells attached on the surface and held together by self-produced extracellular matrix. The biofilm matrix is composed of hydrated extracellular polymer substances (EPS) that form their immediate environment (Flemming and Wingender 2010). The EPS forming the matrix are mainly polysaccharides, proteins, nucleic acids, and lipids. Together, the components of the EPS facilitate the interaction between the cells with the surface and with one another to form a stronger structure of a biofilm and confer mechanical stability to the biofilm.

Biofilm formation is a survival strategy that offers protection to bacterial cells growing in it from environmental stresses such as desiccation, UV radiation, host defense responses, and antimicrobial agents. As a result, formation of biofilms and their inherent resistance to antimicrobial agents contribute majorly to persistent and chronic bacterial infections by human pathogens (Costerton et al. 1999; Kostakioti et al. 2013; Singh et al. 2017). Similarly, biofilm-

forming plant pathogens are also difficult to eradicate and have a better survival chance than their planktonic (free-living) cells (Bogino et al. 2013; Danhorn and Fuqua 2007; Morris and Monier 2003; Rudrappa et al. 2008).

The process of biofilm formation is complex and highly regulated through the production and response of bacterial cells to environmental and cellular signals, and the expression of genes required for survival and maintenance of the biofilm (Wolska et al. 2016). To form biofilms, bacteria switch from free-living mode called planktonic phase to sessile lifestyle by associating with surfaces. The biofilm formation process has four major stages that involve: (i) attachment to the surface, (ii) development of micro-colonies, (iii) maturation of a biofilm structure, and (iv) dispersal. The bacteria in biofilms show phenotypic differentiation compared to their planktonic counterparts. These changes observed in each stage of biofilm development are facilitated by the transcription of genes that occurs in these phases of the bacterial life cycle (Wolska et al. 2016). Biofilm formation is described, therefore, as a process of adaptation and changing genetic regulation (Jefferson 2004; Wolska et al. 2016).

1.2.1. Stages of Biofilm Formation

1.2.1.1. *Attachment to Surfaces*

Attachment is a crucial step for controlling biofilm formation and subsequent chronic and persistent infections. Surface attachment occurs in two phases i.e. reversible and irreversible attachment. During reversible attachment, surface-associated bacteria sample the niche surface before making it a permanent residence. During this early biofilm formation phase, bacteria can still detach from the surface or can commit to irreversible attachment thereby forming a biofilm. The commitment of bacterial cells to irreversible attachment is a crucial step in biofilm formation and is characterized by the establishment of initial surface colonizers that later become the foundation upon which the mature biofilm is built (Caiazza and O'Toole 2004). A number of cell surface components drive the attachment of bacteria to surfaces including surface appendages and extracellular polymeric substances (EPS) (Flemming et al. 2007; Koczan et al. 2011). Surface appendages such as flagella and pili may serve as adhesins that facilitate the attachment of bacteria to surfaces and initial stages of biofilm development (Friedlander et al. 2013; Heindl et al. 2014; Koczan et al. 2011; O'Toole and Wong 2016; Petrova and Sauer 2012; Shi and Sun 2002). For

example, with flagella, bacteria swim towards the surfaces which also facilitate the rotation of bacteria on the surface, while type IV pili (T4P)-mediated twitching motility drives the micro-colony formation processes through surface-associated twitching (Friedlander et al. 2013; Merz et al. 2000; Petrova and Sauer 2012). In most bacteria, T4P and flagella motilities are required for optimal biofilm formation (Guttenplan and Kearns 2014; Koczan et al. 2011; O'Toole and Kolter 1998). In many pathogens, the loss of flagella has been shown to reduce attachment and biofilm formation, indicating that initial attachment to the surfaces is fundamental to the development of a biofilm. Mutations in *Xanthomonas axonopodis* pv. *citri fliC* (flagellin) and the *flgE* (hook) genes showed that the flagella-dependent motilities are required for the initial attachment of the cells to both synthetic and plant leaf surfaces (Malamud et al. 2011). Recently, a mutation in *X. axonopodis* pv. *glycines* flagella hook protein encoded by *flgK* was shown to produce a non-flagellated phenotype with defects in swimming motility and biofilm formation (Athinuwat et al. 2018). Previously, similar results were reported for the plant pathogen *Pantoea ananatis* (Weller-Stuart et al. 2017). Deletion of the *fli*, *flh*, and *flg* gene clusters in *Erwinia amylovora* also confirmed the importance of the flagella in attachment and biofilm formation (Koczan et al. 2011). Following reversible attachment, bacteria can then commit to the differentiation process leading to biofilm formation characterized by production of EPS and aggregation of cells.

1.2.1.2. *Micro-colony Formation*

The initial biofilm development occurs through three independent mechanisms i.e. (i) binary division that allows the formation of clusters; (ii) recruitment of planktonic cells from the bulk liquid to the development of a biofilm; and (iii) redistribution of attached cells by surface motilities including swarming, gliding, creeping and or twitching depending on the substratum (see review Stoodley et al. 2002). It is apparent that T4P-mediated twitching motility facilitates biofilm formation on surfaces and is required during the irreversible attachment stage (Klausen et al 2003). The role of T4P in biofilm development is centered on the development of colonies by adherent cells through twitching motility (Mattick 2002). The initial studies on pilus retraction showed that T4P filaments retract, generate substantial force, and mediate cell movement on surfaces (Merz et al. 2000). Genetic characterization of the factors facilitating T4P-dependent twitching motility shows that this process requires the function of retraction ATPases encoded within the *pil* operon

(Mattick 2002; Merz et al. 2000; Whitchurch et al. 1991; Whitchurch and Mattick 1994). Two of the most studied retraction ATPases in bacteria are PilT and PilU. Studies have shown that mutation in the genes encoding these proteins result in non-twitch phenotypes with varied levels of T4P expression ranging from no-pili to hyper-piliated (Bahar et al. 2009; Burdman et al. 2011; Chiang et al. 2008; Hockenberry et al. 2016; Liu et al. 2001; Rosenberg et al. 2018; Weller-Stuart et al. 2017; Wolfgang et al. 1998). Mutations in *pilT* and *pilU* genes from plant pathogenic species have shown diverse phenotypic expression of pili. As a result, mutations in these retraction ATPases also affect the size of the biofilm formed. *Acidovorax citrulli pilT* mutants are hyperpiliated but express a twitch-minus phenotype and are unable to form biofilms (Bahar et al. 2009; Rosenberg et al. 2018). Interestingly, *A. citrulli pilU* mutants express neither pili nor twitching phenotype and they are unable to form biofilms (Rosenberg et al. 2018). *Pantoea ananatis pilT* mutants are piliated, have reduced twitching motility but form more dense biofilms than those formed by the wild type (Weller-Stuart et al. 2017). Although these results appear to be dissimilar, they, however, still suggest that T4P are required for twitching motility and biofilm formation. Because T4P assembly (Figure 1.2) also requires the function of a number of proteins, studies have shown that these genes are indispensable for the assembly of T4P in different species. Strains carrying a mutation in some of the major T4P assembly genes such as *pilA*, *pilC*, *pilD*, *pilM*, *pilN*, *pilO*, *pilP*, and *pilQ*, fail to express the extracellular pilus and some tested so far, also do not twitch on surfaces and form reduced biofilms (Bahar et al. 2009; De La Fuente et al. 2007; Graupner et al. 2000; Kang et al. 2002; Li et al. 2007; Liu et al. 2001; Meng et al. 2005; Roine et al. 1998; Rosenberg et al. 2018; Weller-Stuart et al. 2017). Mutations in the pilus extension ATPase PilB and the adhesion protein PilY1 also produce strains that are deficient in T4P expression and reduced in twitching motility and biofilm formation (De La Fuente et al. 2007; Li et al. 2007; Meng et al. 2005; Rosenberg et al. 2018).

It is, therefore, apparent that in these bacterial species, T4P assembly, expression, and function is required for biofilm formation. As described above, the presence of functional T4P drives the twitching motility process critical for micro-colony development, the vital precursor stage leading to the maturation of a biofilm.

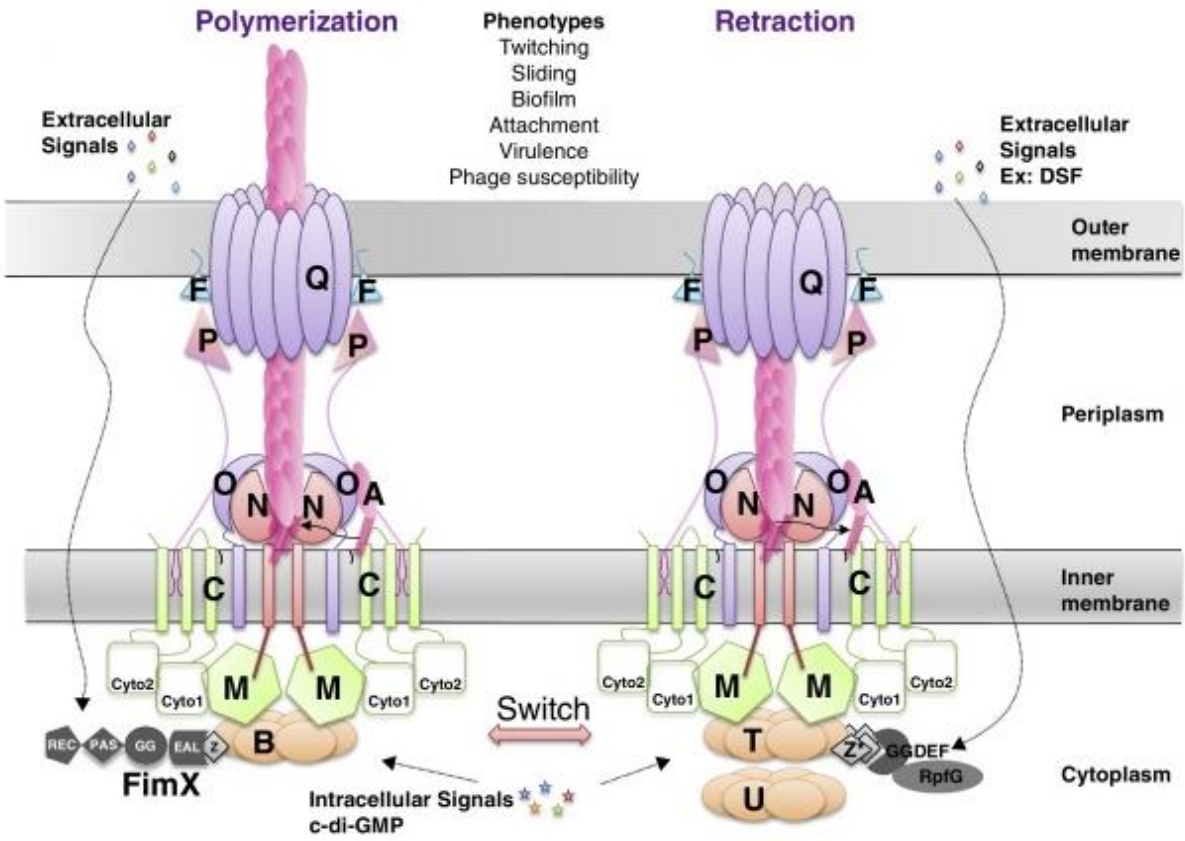

Figure 1.2. Model showing type IV pilus (T4P) complex organization and regulation in *Xanthomonas* species (Dunger et al. 2016). The genes required for the assembly and function of T4P structure are shown as letters for *pilA*, *pilB*, *pilC*, *pilF*, *pilM*, *pilN*, *pilO*, *pilP*, *pilQ*, *pilT*, and *pilU*. The picture shows the regulation of T4P polymerization and retraction by *pilB*, *pilT*, and *pilU* coupled with diffusible signal factor (DSF) extracellular signaling and intacellular signaling involving c-di-GMP, FimX, PilZ, RpfG, and GGDEF proteins (refer to section 1.2.2.). The picture also lists T4P-related phenotypes affected in *Xanthomonas* species.

1.2.1.3. *Biofilm Maturation*

A mature biofilm is a 3-dimensional structure of aggregated cells attached to a surface and enclosed in a biofilm matrix. The biofilm maturation stage is dependent upon attachment and micro-colony development. The major components of a mature biofilm are cells, biofilm matrix, water, and channels for the passage of nutrients and toxic waste products. Following irreversible attachment and micro-colony formation, bacteria secrete EPS to form a biofilm matrix, which serves as the housing of the cells growing in a biofilm (Flemming et al. 2007). The biofilm cells grow embedded in the EPS matrix, which facilitates the interaction between the cells with the surface and with one another to form a stronger structure of a biofilm. EPS confer mechanical

stability and have a role in water retention and nutrient availability. The components of the biofilm matrix include polysaccharides, proteins, glycoproteins, glycolipids, extracellular DNA (e-DNA).

The EPS matrix interacts with the environment, for example, by attaching biofilm cells to surfaces and through its sorption properties, thereby providing nutrients for biofilm cells (Flemming 2016). Evidence of EPS production by plant pathogenic bacteria including *Pseudomonas syringae* pv. *syringae*, *Acidovorax avenae* subsp. *avenae* RS1, *Ralstonia solanacearum*, *Xylella fastidiosa*, and *Xanthomonas* species has been observed in the past (Bianco et al. 2016; Denny 1990; Killiny et al. 2013; Penaloza-Vázquez et al. 1997; Singh et al. 2006; Zhang et al. 2017). In some of these bacterial species, EPS is highly associated with virulence (Bianco et al. 2016; Denny 1990; Killiny et al. 2013; Singh et al. 2006). *Xanthomonas* species synthesize a specialized EPS, referred to as xanthan, which contributes to bacterial stress tolerance and supports biofilm formation and attachment to plant surfaces (Bianco et al. 2016; Yun 2006). The *Xanthomonas axonopodis* pv. *citri gumB* mutant, defective in the production of the EPS xanthan, did not form structured biofilms on either abiotic or biotic surfaces (Rigano et al. 2007). As a result, the same mutant also showed reduced growth and survival on leaf surfaces and reduced disease symptoms (Rigano et al. 2007). Similarly, the EPS of *X. fastidiosa* also known as fastidian gum is essential for biofilm formation, virulence, and vector transmission (de Souza et al. 2013; Killiny et al. 2013). Mutation in *X. fastidiosa* EPS biosynthesis genes *gumD* and *gumH* produces mutant strains that are defective in EPS production, attachment, biofilm formation and are avirulent in plant hosts (Killiny et al. 2013). *Pantoea* (*Erwinia*) *stewartii* requires EPS to colonize and cause disease in plants (Braun 1990). Herrera et al (2008) also demonstrated that *P. stewartii* requires EPS and motility to induce wilt on susceptible hosts. As previously mentioned in section 1.1.3, there are very few known pathogenicity factors of *X. ampelinus*. The role played by EPS in pathogenicity and biofilm formation and the genes regulating EPS production in this bacterium are not yet known, but should be investigated because of its importance in plant-microbe interactions, virulence, and in the establishment of biofilms on host surfaces.

1.2.1.4. *Biofilm Dispersal*

Dispersal is the last stage of biofilm development and is characterized by the detachment of cells from the biofilm. Bacteria growing in a biofilm complete their cycle by detaching from the biofilm

and floating back into the environment to colonize new surfaces (Kaplan 2010). A basic mechanism of biofilm dispersal is the production of extracellular enzymes such as cellulases, dispersin B, alginate lyase, and mannosidases among many that degrade adhesive components in the biofilm matrix (Fleming and Rumbaugh 2017). These enzymes degrade the biofilm matrix encasing the bacterial cells within the biofilm colony, thus initiating the detachment of cells from the colony releasing them into the environment.

In human pathogens, biofilm dispersal plays an important role in the transmission of bacteria from environmental reservoirs to human hosts and in the spread of infection within the host (Kaplan et al. 2003). Although there is limited evidence of this fact with regard to plant pathogenic bacteria, their ability to form and detach from biofilms may equally have considerable implications for the completion of their disease cycle. Dow et al. (2003) showed that biofilm dispersal in *X. campestris* pv. *campestris* is required for full virulence in plants indicating that the disease cycle of plant pathogens could also be similar to that of well-studied human pathogens.

1.2.2. Regulation of Biofilm Formation

During biofilm formation, a number of environmental signals also regulate attachment to surfaces including nutrient availability and quorum sensing (Stanley and Lazazzera 2004). The cells in a biofilm alter their physiological processes in response to environmental conditions via signaling pathways. Differentiation involves obvious changes driven by gene expression altering functions in the cell resulting in a different phenotype. Different biofilm phenotypes have been described over the years as observed in many pathogens. Malamud et al. (2013) identified 23 genes that were previously not associated with biofilm formation in *X. citri* subsp. *citri*. In addition to biofilm formation, these genes were also required for EPS production, motility, and cell surface structures critical for the biofilm formation process (Malamud et al. 2013). A proteomic approach to characterize *X. axonopodis* pv. *citri* biofilms also identified 53 differentially expressed proteins during mature biofilm formation, which are involved in major cellular functions and signaling (Zimaro et al. 2013). Similar phenotypic differentiation has also been observed in *X. fastidiosa* species (de Souza et al. 2004; Silva et al. 2011). Signaling is therefore a very important determinant in the regulation of biofilms.

The biofilm formation process requires both environmental and cellular information processing. Regulation of the biofilm formation process in many bacterial species involves quorum sensing, c-di-GMP, and sRNAs. Cell-to-cell communication often referred to as quorum sensing (QS), regulates gene expression in response to fluctuations in cell-population density. Current research shows that c-di-GMP is a general key regulator of the biofilm formation cycle (see review Hengge 2009). High internal levels of c-di-GMP induce the production of adhesins and extracellular matrix components that enable bacteria to form biofilms, whereas low c-di-GMP levels downregulate the production of adhesins and extracellular matrix components and lead biofilm bacteria into dispersal to undertake a planktonic mode of growth. In addition to these two regulators, biofilm formation is also regulated by small RNAs that modulate regulatory networks enabling concentration-specific responses, by sequestering, antagonizing or activating regulatory proteins in response to environmental cues, or by directly affecting the synthesis of proteins promoting/disfavoring the formation of biofilms (Chambers and Sauer 2013).

1.2.2.1. *Quorum Sensing (QS)*

Cell-to-cell communication and community behaviors are critical for the successful interaction of pathogens with their hosts. Cell-to-cell communication or quorum sensing (QS), regulates gene expression in response to the changes in cell-population density. Quorum sensing bacteria produce and release chemical signal molecules called auto-inducers that increase in concentration as a function of cell density. Upon the detection of a minimal threshold stimulatory concentration of an auto-inducer, bacteria begin to alter their gene expression (Miller and Bassler 2001). QS-mediated gene expression is usually a response necessary for the continued success of the pathogen inside the host. Since the 1980s, several types of QS signals have been identified, which are associated commonly with different types of QS mechanisms. These chemical QS signals are produced by specific enzymes and are detected by specific receptors in the cells. To date, the most studied QS signals are the N-acyl homoserine lactones (AHL), signal diffusible factor (DSF), and methyl 3-hydroxypalmitate (3-OH PAME) among others (Papenfort and Bassler 2016). In some pathogens, these QS signals directly regulate the process of biofilm formation and/ or dispersal. For example, the AHL QS signals negatively regulate biofilm formation in *A. citrulli*. Mutations in *A. citrulli luxI* and *luxR* homologs *aacI* and *aacR*, respectively induced biofilm formation (Fan

et al. 2011; Wang et al. 2016). Similarly, DSF QS signals also induce biofilm dispersal in *Xanthomonas* species (Dow et al. 2003).

Among the QS signaling molecules known thus far, DSF is the most studied in plant pathogens such as those belonging to the *Xylella* and *Xanthomonas* genera (Barber et al. 1997; Barel et al. 2015; Chatterjee et al. 2008; de Souza et al. 2013; He et al. 2010; Ionescu et al. 2013; Li et al. 2019; O'Connell et al. 2013; Torres et al. 2007; Zhao et al. 2011). In these species, the DSF signaling system mediates a regulatory system required for pathogenicity encoded within the *rpf* (regulation of pathogenicity factors) gene cluster (Barber et al. 1997; Tang et al. 1991). The *rpf* genes are required for DSF signal synthesis and perception, adaptation, virulence, and biofilm formation and dispersal (Figure 1.3) (An et al. 2014; Andrade et al. 2006; Chatterjee et al. 2008; Deng et al. 2012; He et al. 2006; Ionescu et al. 2013; O'Connell et al. 2013; Ryan et al. 2015; Tang et al. 1991; Wang et al. 2016).

In plant-pathogenic *Xanthomonas* species, the synthesis of DSF is totally dependent on RpfF, which has amino acid sequence relatedness to enoyl CoA hydratase and is partially dependent on RpfB, which is a long-chain fatty acyl CoA ligase (Barber et al. 1997). DSF sensing and signal transduction involves a two-component system comprising the sensor RpfC and regulator RpfG (Slater et al. 2000). Recently, a second DSF sensor, RpfS, of *X. campestris* pv. *campestris* was discovered (An et al. 2014). Mutations in some of these genes in *X. campestris* pv. *campestris* shows that controlled synthesis of the DSF cell-to-cell signal is required for biofilm formation and virulence (Torres et al. 2007). In this study, an *rpfF* mutant (DSF-minus) and *rpfC* mutant (DSF overproducer) formed only unstructured arrangements of bacteria that did not develop into a biofilm suggesting that DSF signaling is balanced during biofilm formation (Torres et al. 2007). In addition to biofilms and virulence, the DSF-mediated QS also plays a central role in coordinating gene expression of *Xylella* and *Xanthomonas* species (An et al. 2014; Cursino et al. 2015; Guo et al. 2012). Some of the major processes that are regulated by DSF signaling system are motility, colonization, biofilm dispersal, extracellular enzyme production, toxin production, vector transmission, and EPS production (Chatterjee et al. 2008; He and Zhang 2008; Malamud et al. 2011; Ryan et al. 2007; Wang et al. 2012). Moreover, QS signal sensing is coupled to intracellular regulatory networks through a second messenger cyclic-di-GMP and a global regulator Clp (Figure 1.3.).

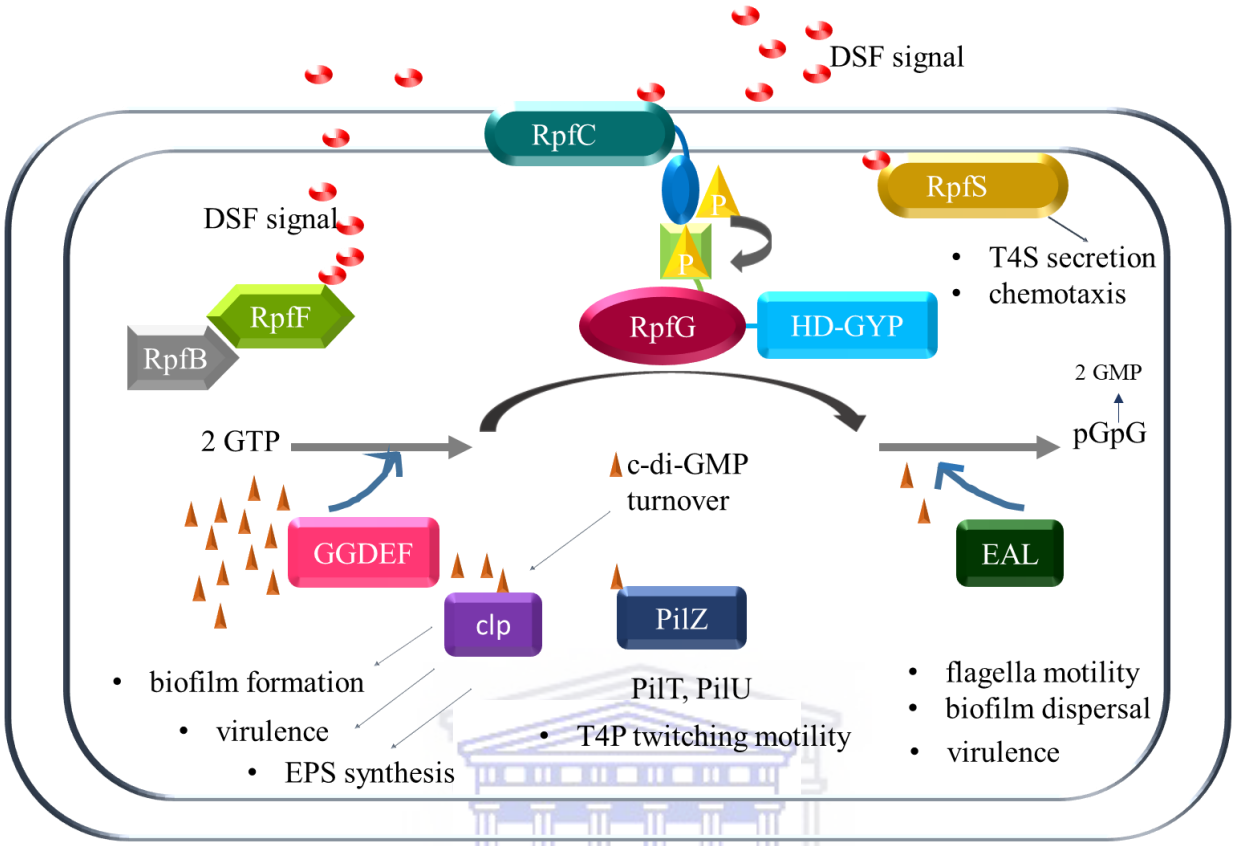

Figure 1.3. Schematic representation of DSF signaling pathway and regulation of cellular functions (modified from Dow et al. 2006; Ryan 2013). Under high cell population density, bacteria synthesize a small diffusible signal factor (DSF) signal molecule through RpfF and RpfB (Barber et al. 1997). The bacteria sense the presence of the DSF signaling molecule through the RpfC sensor which form a part of the RpfC/RpfG two-component sensory transduction system (Tang et al. 1991). Upon its interaction with the DSF signal, RpfC, a hybrid membrane sensor kinase, phosphorylates RpfG. The function of the response regulator RpfG is to regulate the intracellular levels of the cyclic di-GMP (Ryan 2013). While the GGDEF domains of the diguanylate cyclases catalyze the synthesis of c-di-GMP from 2 GTP, the HD-GYP and EAL domains of the phosphodiesterase catalyze the hydrolysis to pGpG and 2 GMP. The phenotype exhibited in response to the levels of c-di-GMP present in the cell are dependent on the regulators that respond to levels by activating or repressing the transcription of genes to influence the phenotype (Lu et al 2012). Clp is a cyclic di-GMP receptor and a global transcription regulator that regulates expression of specific genes such as those required for biofilm formation, virulence, and extra polymer substances (EPS) (Ryan 2013). Clp links the Rpf/DSF signaling system to the expression of genes for specific phenotypes (Chin et al. 2010). The function of the PilZ domain, also in the regulation of twitching motility, requires the HD-GYP:GGDEF:PilZ complex studied in *Xanthomonas* species (Dunger et al. 2016; Ryan 2013). Reduced levels of c-di-GMP trigger biofilm dispersion and motility in through Rpf/DSF signaling (Dow et al. 2003). RpfS, predicted to have multiple Per/Arnt/Sim (PAS) domains, is a second sensor for DSF in *Xanthomonas campestris* pv. *campestris*, which controls the genes involved in type IV secretion and chemotaxis (An et al. 2014).

1.2.2.2. *Cyclic diguanylate (c-di-GMP)*

Cyclic di-GMP is a second messenger used in signal transduction by a wide variety of bacteria. Cyclic di-GMP represents a signaling system that regulates many bacterial behaviors and is of key importance for driving the switch between motile and biofilm existence of bacterial cells (Valentini and Filloux 2016). Cyclic di-GMP is produced by enzymes containing GGDEF domains (diguanylate cyclases [DGCs]) and is hydrolyzed by those containing either EAL or HD-GYP domains (phosphodiesterases [PDEs]) that are found in many species of bacteria in large numbers (Hengge 2009; Valentini and Filloux 2016). The GGDEF, EAL, and HD-GYP domain-containing proteins affect cell differentiation and multicellular behavior as well as interactions between the microorganisms and their hosts and other phenotypes. The effect of these proteins in controlling multicellular behaviors is dependent on the internal c-di-GMP modulation. The level of c-di-GMP in the cells are modified by the rate of its synthesis and degradation, thereby, causing the cells to switch from one lifestyle to the other (Valentini and Filloux 2016). The correlation between high c-di-GMP concentration in the cell and biofilm formation or between low c-di-GMP levels and motility has been demonstrated in several bacterial species, e.g. *Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella enterica* serovar Typhimurium (Simm et al. 2004).

In *Xanthomonas campestris* pv. *campestris*, c-di-GMP participates as an intracellular signaling molecule that couples extracellular DSF signaling to the expression of virulence traits (Dow et al. 2003; 2006). The detection of DSF signal by the sensor RpfC leads to the activation of response regulator RpfG, which activates virulence gene expression by degrading c-di-GMP (Chatterjee et al. 2008; He and Zhang 2008; Tao et al. 2010). Similarly, in *X. fastidiosa*, the DSF-dependent signaling involves the modulation of cyclic di-GMP levels (Chatterjee et al. 2008). The decrease of c-di-GMP levels result from the accumulation of DSF as cell density increases, leading to phenotypic transition from a planktonic state to a biofilm state (Chatterjee et al. 2008, 2010). Furthermore, this transition is required for the pathogenesis of *X. fastidiosa* as bacteria adhere to the insects for transmission from one plant to another (Chatterjee et al. 2010).

In many bacterial pathogens, the second messenger c-di-GMP stimulates the production of an EPS matrix to shield bacteria from harmful external factors such as antimicrobial agents, toxins, and host defense responses. Past research shows that c-di-GMP acts as an allosteric activator of cellulose synthase and poly-B-1,6-N-acetylglucosamine among other extracellular matrix

components that form the EPS (Ross et al. 1987; Steiner et al. 2013). The evidence of this fact has also been seen in plant pathogens belonging to *Xylella* and *Xanthomonas* genera studied thus far. *Xylella fastidiosa eal* and *rpfF* mutants are impaired in EPS production and form reduced biofilms compared to the wild type strain (de Souza et al. 2013). The *X. fastidiosa*, PD1671 gene, containing the degenerate c-di-GMP GGDEF/EAL domains, is involved in biofilm formation, seemingly through regulation of *gum* gene expression and therefore EPS production (Cursino et al. 2015). A two-component system, PdeK/PdeR, of *Xanthomonas oryzae* pv. *oryzae* regulates c-di-GMP turnover, virulence, and EPS production by this bacterium (Yang et al. 2012). Recently, a response regulator, TriP, of *X. oryzae* pv. *oryzae* was also reported to regulate virulence and EPS production via its interaction with PdeR (Li et al. 2019).

Furthermore, c-di-GMP is implicated in the regulation of cell motilities during biofilm development. Cyclic di-GMP plays a critical role in the regulation of motility of many bacteria, being most studied in *P. aeruginosa*. It is apparent that the transition from planktonic to biofilm state undergone by bacteria requires the regulation of cell motilities. In *P. aeruginosa*, biofilm formation and swarming motilities of the flagella are inversely regulated (Caiazza et al. 2007). Guttenplan and Kearns (2014) reviewed the regulation of flagellar motility during biofilm formation. One of the ways in which cells coordinate flagellar and biofilm gene expression is by controlling the transcriptional regulator FleQ, which activates flagellar genes and represses the expression of the *pel* operon that is required for EPS biosynthesis (Baraquet et al. 2012; Guttenplan and Kearns 2014). The mechanism of the FleQ functional switch is associated with its interactions with c-di-GMP as seen in *P. aeruginosa* (Baraquet et al. 2012; Hickman and Harwood 2008). Likewise, *X. campestris* pv. *campestris* flagella motility, biofilm and virulence are also regulated through c-di-GMP modulation (Tao et al. 2014; Yang et al. 2009).

T4P-mediated motilities, required during early stages of biofilm attachment and micro-colony formation are also regulated by signaling systems. In *P. aeruginosa*, approximately 40 proteins have been identified as necessary for T4P-related functions including pilus structure and assembly, function, and chemosensory pathways that control activity in response to environmental signals (Huang et al. 2003; Mattick 2002; Pelicic 2008). *Pseudomonas aeruginosa* also expresses a two-component transcriptional regulatory system consisting of the sensor kinase, PilS, and a response regulator, PilR, which is activated by environmental signals. This signal transduction system is

involved in transcription of *pilA*, the major T4P pilin subunit (Hobbs et al. 1993; Köhler et al. 2000). Recently, response regulator, PilR, was identified and characterized in *X. oryzae* pv. *oryzicola* (Zhang et al. 2018). Functional studies following mutation analysis showed that *pilR* mutants show reduced virulence on plant hosts (Zhang et al. 2018). In *Lysobacter enzymogenes* the PilR function in controlling twitching motility is associated with c-di-GMP (Chen et al. 2017). Similarly, in *Myxococcus xanthus* modulation of c-di-GMP levels is important for both T4P-dependent motility and cell agglutination (Skotnicka et al. 2015). Therefore, it is likely that *X. ampelinus* regulates the biofilm formation and motilities through the modulation of intracellular c-di-GMP levels.

Another interesting protein necessary for T4P assembly and twitching motility is PilZ, which is widely distributed among *Xanthomonas* species. The PilZ protein was originally identified as necessary for T4P assembly (Alm et al. 1996). However, a large and diverse family of bacterial PilZ homology domains have been identified some that are implicated in signaling pathways that control twitching motility, biofilm formation, and virulence. For example, *X. axonopodis* pv. *citri* PilZ interacts with the T4P extension ATPase, PilB, and the T4P regulatory protein, FimX, which carries a phosphodiesterase (EAL) domain (Guzzo et al. 2009). Interactions between the PilB, PilZ, and FimX explained in this work proves the direct interactions of these proteins with the T4P machinery that mediates twitching motility, and indicate that this type of motility is highly regulated in bacteria (Figure 1.2). The evidence of the involvement of c-di-GMP in the regulation of T4P assembly was later provided through biochemical and structural analysis of the *X. axonopodis* pv. *citri* PilZ-FimX-c-di-GMP complex (Guzzo et al. 2013). Recently, formation of a HD-GYP-GGDEF-PilZ domain complex was reported to regulate T4P-dependent twitching motility function in *X. campestris* pv. *campestris* (An and Tang 2018).

Cyclic di-GMP is not only critical for biofilm formation but is also involved in biofilm dispersal in *Xanthomonas* species (Dow et al. 2003). Biofilm dispersal is the last stage of the biofilm formation cycle and it involves the detachment of single cells from the biofilm and return to the bulk fluid. Same as biofilm formation, biofilm dispersal is a genetically programmed response enabling bacterial cells to exit the biofilm in response to particular physiological or environmental conditions. For example, biofilm dispersal could be associated with nutrient starvation, which

triggers lower levels of c-di-GMP in species such as *Pseudomonas putida* (Gjermansen et al. 2010).

Biofilm dispersal in *X. campestris* pv. *campestris* is controlled by regulation of cell-to-cell signals through the RpfF-DSF synthase complex. In *X. campestris* pv. *campestris* DSF QS signal triggered dispersion of biofilm aggregates formed by the *rpfF* mutant strain indicating that in response to the DSF signal, biofilm dispersal occurs, thereby promoting virulence (Dow et al. 2003). Evidence of the involvement of the RpfF-DSF system in regulating the pathogenicity of *X. campestris* pv. *campestris* in response to physiological or environmental changes was initially recorded by Barber et al. (1997). Recently more evidence has surfaced involving the regulation of virulence in other *Xanthomonas* species through this system (Li et al. 2019; Li et al. 2019; Yang et al. 2012).

1.3. Biofilm Formation and Virulence of Plant Pathogens

Biofilm formation accounts for the most success of the pathogen inside the host and subsequently its virulence. The main reason why biofilms are so important is the fact that they can be nearly impossible to eradicate because of their resistant nature. In some cases, biofilms are also associated with persistent infections as seen in *X. fastidiosa* subsp. *pauca* (Muranaka et al. 2012). In many plant pathogens, the ability to form biofilms is associated with the pathogenesis of such pathogens. For example, major components of biofilm formation such as EPS, flagella, and T4P are required for virulence in many bacterial species. Functional studies of specific genes involved in the synthesis of biofilm components show that in the absence of such genes, the bacterial species fail to form biofilms and also have relatively reduced virulence. As mentioned before, bacterial EPS is required for irreversible attachment of bacterial cells to the surface, biofilm maturation, and subsequently virulence. Mutations in EPS biosynthesis genes have shown defects in biofilm formation and virulence in such species. The major mechanisms of *X. axonopodis* pv. *citri* pathogenesis are supported by its ability to attach and be established through biofilm formation on the host tissues (Gottig et al. 2010). Gottig et al. (2010) also reported that a *Xanthomonas* genus characteristic is the synthesis and secretion of an EPS, which plays an important role in epiphytic survival and biofilm formation by the species found in this genus. The factors contributing to biofilm formation and their role in virulence have not been studied in *X. ampelinus* but should be

investigated to gain insights on the virulence factors associated with attachment and biofilm formation in this bacterium.

T4P have also been implicated in the virulence of many plant pathogens including *X. fastidiosa*, *A. citrulli*, *P. syringae* pv. *tabaci*, and *R. solanacearum* (Burdman et al. 2011; Kang et al. 2002; Meng et al. 2005; Rosenberg et al. 2018; Taguchi and Ichinose 2011). T4P-deficient *X. fastidiosa* mutants expressing a twitch-minus phenotype are unable to form biofilms and are inhibited from colonizing upstream vascular regions *in planta* (Meng et al. 2005). *Ralstonia solanacearum* T4P *pilA* mutant cells have a twitch-minus phenotype, and are also reduced in auto-aggregation and virulence (Kang et al. 2002). *Pseudomonas syringae* pv. *tabaci* cells with mutations in T4P assembly genes fail to express T4P, are unable to twitch on semi-solid medium, and have reduced virulence (Taguchi and Ichinose 2011). Similar to these reports, *A. citrulli* T4P mutant cells form reduced biofilms and are reduced in virulence (Bahar et al. 2009; Rosenberg et al. 2018). All these studies conclude that T4P are required for twitching motility, biofilm formation, and virulence.

1.4. Biofilm Formation in Xylem Vessels

A number of phytopathogens enter the plants through different infection sites and eventually colonize and multiply within the xylem vessels. *Ralstonia solanacearum*, *X. oryzae* pv. *oryzae*, *X. campestris* pv. *campestris*, *E. amylovora*, *P. stewartii* subsp. *stewartii*, *Clavibacter michiganensis* subsp. *michiganensis*, *P. syringae* pv. *actinidiae*, and *X. fastidiosa*, are some of the well-studied xylem-colonizing bacteria (Bae et al. 2015). As a biofilm-forming bacterial pathogen, *X. fastidiosa* forms aggregates that occlude the xylem vessels of the plants and subsequently lead to disease manifestation (Tyson et al. 1985). In pathology, vessel blockage is a major contributor to disease development caused by bacteria and that the grapevine xylem sap supports the growth of bacterial species as seen with *X. fastidiosa* (Zaini et al. 2009). In *X. fastidiosa* just like in any other xylem-colonizing pathogens, the disease is associated with a progressive increase in the number of xylem vessels colonized in the plant, eventually reaching a point where water transport is compromised enough to initiate symptoms associated with water stress (Chatterjee et al. 2010).

The plant xylem vessels are known to favor nutrient uptake by bacteria, thereby enabling the development of large population sizes in this environment, which is thought to be nutrient poor

(Zaini et al. 2009). Biofilm development in plant xylem is initiated by the attachment of cells to the inner wall of xylem vessels followed by an aggregation step, which results in the development of mature biofilms that occlude xylem vessels as seen in *X. fastidiosa*, *R. solanacearum*, and *E. amylovora* (Kang et al. 2002; Koczan et al. 2011; Newman et al. 2003). Colonization of the xylem vessels by bacteria is a complex process that requires specialized organelles such as T4P. T4P also facilitate the migration of bacteria in the host plant's vascular system against the direction of the transpiration stream (Meng et al. 2005). Meng et al. (2005) used the fabricated microfluidic chambers to mimic the xylem vessels and showed that *X. fastidiosa* requires T4P to colonize the plants. Scanning electron microscopy of melon xylem vessels following seed-transmission assays with *Acidovorax avenae* subsp. *citrulli* M6 illustrated colonization of xylem vessels by M6 cells and biofilm development on the xylem tissue (Bahar et al. 2009). Colonization of the grapevine leaf xylem vessels and biofilm formation by *X. fastidiosa* was also documented using scanning electron microscopy to characterize the Pierce's disease development in plants (Tyson et al. 1985).

1.5. Summary of the Research Progress on Biofilm Formation by Plant pathogens

A large proportion of research has been projected towards studying biofilm formation in plant pathogenic bacteria and its association with bacterial virulence. Biofilm formation by a number of bacterial phytopathogens have been studied over the years including *Xanthomonas*, *Pseudomonas*, *Erwinia*, *Ralstonia* and *Xylella* species (Koczan et al. 2008; Marques et al. 2002; Mori et al. 2015; Rigano et al. 2007; Tomihama and Nishi 2007).

A number of different methods have been used to identify and characterize genes involved in biofilm formation. Transposon mutagenesis and microarrays have long been employed in biofilm formation studies and have helped in revealing novel genetic determinants and regulation mechanisms of biofilm formation (Li and Wang 2011; Li et al. 2007; Rosenberg et al. 2018). In addition, determining the biofilm proteome has also recently gained popularity (Silva et al. 2011; Zimaro et al. 2013). The proteins expressed during the different stages of biofilm formation have also been identified and characterized to understand the physiological changes undergone by biofilm cells. This then gives insight into the functions required by the pathogen to cause the disease in plants, thereby opening a channel to finding ways of attenuating such functions for disease control purposes. Microarrays and Liquid chromatographic mass spectrophotometry (LC-MS) techniques have been employed by many research groups to study the differential expression of genes and proteins expressed by the biofilm and planktonic cells (de Souza et al. 2004; Silva et al. 2011; Zimaro et al. 2013). Also, much focus has been set on the attachment and motility structures contributing to biofilm formation (Attinuwat et al. 2018; Bahar et al. 2009; Burdman et al. 2011; Dunger et al. 2014; Koczan et al. 2011; Li et al. 2007; Weller-Stuart et al. 2017). Studying these complex structures requires the characterization of their assembly and functional regulatory genes. Flagella and pili have received more attention due to their roles in motility and surface attachment. In addition to proteomic and genomic approaches, few more techniques such as transcriptomics, metagenomics, metabolomics, and biofilm imaging have been employed to study biofilms (Franklin et al. 2015).

1.6. Research Objectives

Xylophilus ampelinus is a bacterial blight-causing pathogen believed to inhabit and form biofilms in the xylem vessels of infected grapevines. *Xylophilus ampelinus* has long been a threat in the grapevine industry resulting in reduced yield due to destruction and shortened lifespan of the vines. Because of the economic importance of grapevines, this destructive pathogen qualifies for further investigation. The biofilm formation process of this pathogen and it role in virulence is still unknown regardless of its significance in disease manifestation. Therefore, this study is aimed at characterizing the biofilm formed by *X. ampelinus*.

There are two aspects to this study: (i) Because biofilm formation is a response to environmental signals, resulting in changes in protein expression, the proteins showing differential expression during the micro-colony formation stage (initial biofilm) and the mature biofilm stage when compared to the free-living (planktonic) cells was examined, following the identification *in vitro* of the major stages of biofilm formation by *X. ampelinus*; (ii) this study also sought to investigate the interaction of the cells with biotic and abiotic surfaces by studying the function of the attachment and motility organelles, the type IV pili (T4P). By cloning six major T4P assembly and function genes (*pilA, pilB, pilC, pilD, pilQ,* and *pilU*), the role of T4P in *in vitro* and *in planta* biofilm formation, twitching motility, and virulence in grapevine was elucidated.

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2. CHAPTER 2: Analysis of the biofilm proteome of grapevine pathogen, *Xylophilus ampelinus*

2.1. INTRODUCTION

Xylophilus ampelinus is the causal agent of bacterial blight disease of grapevines. The disease is known to occur in certain vine-growing areas of Europe and the Mediterranean region, Japan, and South Africa (Erasmus et al. 1974; Grasso et al. 1979; Komatsu and Kondo 2015; Mathee et al. 1970; Panagopaulos 1969; Prunier et al. 1970). This destructive disease of the vines is characterized by the formation of necrotic lesions, cracks and cankers on shoots, angular reddish-brown lesions, and discoloration on leaves, blackening of young bunches during flowering, and delayed bud burst resulting in vines with stunted appearance. To date, chemicals have failed to control the disease. The initial infections may occur through wounds and natural openings, and once in the plant host, the bacterium is mainly present in xylem vessels of the plants, presumably as biofilms (Grall and Manceau 2003). However, the genetic factors contributing to the pathogenicity of *X. ampelinus* are still poorly understood. A previous study investigating molecular and proteomic profiles of *X. ampelinus* was performed to identify the possible pathogenicity factors of this bacterium (Sevillano 2014). A number of proteins responsible for adaptation, virulence, metabolism, signaling, translation, and motility were identified (Sevillano 2014; Sevillano et al. 2014).

Biofilm formation is one of the most studied pathogenicity factors in bacteria and is beneficial for the fitness and survival of a pathogen (Flemming et al. 2007; Johnson 2008). A biofilm is defined as a well-structured consortium of bacterial cells attached to surfaces and surrounded by self-secreted matrix. The matrix surrounding the bacteria in a biofilm provides protection from a number of stressful environmental factors including extreme temperatures and pH, UV light, antimicrobial agents, and desiccation. As a result, cells growing in a biofilm show increased resistance to diverse forms of stress compared to their free-living counterparts (Bogino et al. 2013; Donlan 2002; Flemming et al. 2007). The major components of a biofilm matrix such as flagella, pili, polysaccharides, lipids, nucleic acids, and proteins are collectively known as extracellular polymeric substances (EPS). The EPS is required for the development, maturity, and maintenance of a biofilm and it also provides mechanical stability of a biofilm (Flemming and Wingender 2010).

Functional characterization of bacterial EPS shows that mutations in genes responsible for the production of EPS produce less-virulent phenotypes that are also deficient in biofilm formation (Killiny et al. 2013; Koczan et al. 2009; Rigano et al. 2007).

Biofilm formation is widely associated with the virulence of pathogenic bacteria. In many plant pathogenic bacteria, e.g. *Ralstonia solanacearum*, *Xanthomonas axonopodis* pv. *citri*, *Erwinia amylovora*, *Pantoea stewartii* subsp. *stewartii*, *Pseudomonas syringae* pv. *actinidiae*, *Pectobacterium carotovorum*, and *Xylella fastidiosa*, biofilm formation is an important factor in the disease cycle inside their hosts (Bogino et al. 2013; Castiblanco and Sundin 2016; Ghods et al. 2015; Koczan et al. 2009; Koutsoudis et al. 2006; Kubheka et al. 2013; Lowe-Power et al. 2018; Marques et al. 2002; Ramey et al. 2004; Rigano et al. 2007). The work that was done on the pathogen, *R. solanacearum*, shows that *in planta* biofilm formation is required for fitness and survival of the populations against the effects of the moving forces of nutrient-streams inside the plants (Lowe-Power et al. 2018). In some cases, biofilm formation is required for epiphytic survival before disease formation inside the host (Rigano et al. 2007). Also, mutations resulting in a biofilm-deficient phenotype, usually translate to reduced or suppressed virulence (Kang et al. 2002; Koczan et al. 2009; Rigano et al. 2007; Rosenberg et al. 2018; Torres et al. 2007).

Attachment to the host, by pathogens, is the first step to pathogenicity. Establishment of a pathogen at the site of infection and inside host tissues promotes virulence and provides protection against host defense responses (Danhorn and Fuqua 2007). In plants, both the phloem and the xylem are subject to colonization by phytobacteria. One of the most studied xylem-colonizing pathogens is *Xylella fastidiosa*, which causes major diseases such as Pierce's disease of grapevines, citrus variegated chlorosis and olive quick decline syndrome (Hopkins and Purcell 2002; Saponari et al. 2017). This bacterium forms biofilms in the xylem vessels of the plant and occludes the vessels, preventing the flow of nutrients and water to the lateral parts of the plants, a phenomenon that was also observed for *P. carotovorum* (Kubheka et al. 2013; Tyson et al. 1985). Analysis of the biofilm proteome and gene expression profile of *X. fastidiosa* revealed that during biofilm formation of this bacterium, a number of genes involved in surface attachment were induced (de Souza et al. 2004; Silva et al. 2011). The analysis of *X. fastidiosa* genome sequence also revealed a number of fimbrial and afimbrial proteins required for attachment that are expressed in *X. fastidiosa* biofilms (Carseta et al. 2010). In the same light, the analysis of the causal agent of citrus canker,

Xanthomonas axonopodis pv. *citri*, biofilm proteome also revealed the overexpression of proteins involved in adherence (Zimaro et al. 2013). These reports depict the importance of surface attachment during biofilm formation, surface colonization, and the establishment of the pathogen inside host plants.

Furthermore, the transition of cells from planktonic to immotile aggregates of a biofilm is a complex and highly regulated process. Generally, the biofilm development process occurs in stages including attachment, micro-colony formation during initial biofilm, mature biofilm, and dispersal or transmission stage. Each of these stages requires the specialized function of specific genes and cellular components and the differences in phenotype between the stages is striking. The proteome analysis of many other bacterial species shows that proteins expressed by bacteria in a biofilm show a very large fold-difference compared to the planktonic cells. This shows the substantial changes undergone by the bacteria to switch between the two lifestyles. The fundamental core of these changes originates from the ability of bacteria to produce and respond to the signals required for their survival. During biofilm formation, the transition may require the regulation of motility and attachment genes. Motility is required for the initial attachment of the cells to the surface but is turned off during biofilm maturation (Amores et al. 2017; Guttenplan and Kearns 2014; Watnick and Kolter 1999). The ability of the cells to switch from the planktonic state to biofilm state is powered by signaling systems including the regulation of secondary messenger cyclic dimeric (3′-5′) GMP (c-di-GMP) intracellular levels (Römling et al. 2013; Ryan 2013). Regulation of signals is a major function in the biofilms that occurs via density-dependent cell-to-cell communication, often termed quorum sensing. Through quorum sensing, decisions regarding the regulation and maintenance of the biofilm community are made. The maturation of biofilms requires enhanced signaling and transcription of many genes required for the regulation of a biofilm. As a result, many biofilm-forming bacteria engage in quorum sensing. *Xylella fastidiosa* is one of the plant pathogens that use cell-to-cell signaling to interact with both insect vectors and its plant hosts (Newman et al. 2004). Other plant pathogens including *P. stewartii*, *R. solanacearum*, *P. syringae*, *Acidovorax citrulli*, and several other species of *Xanthomonas* have also been shown to engage in quorum sensing to coordinate gene expression, and to regulate processes such as adaptation, biofilm formation, virulence, and motility (Andrade et al. 2006; Guo et al. 2012; He and Zhang 2008; Koutsoudis et al. 2006; Kumar et al. 2015; Quiñones et al. 2005;

Torres et al. 2007; Wang et al. 2016; Wang et al. 2016). In addition to these, bacteria use quorum sensing to regulate many other cellular functions and control the response of the cells to environmental stimuli.

The interconnection between biofilm formation, quorum sensing, and virulence remains the focus in gaining insight on pathogenicity and its underlying factors. Therefore, this study was designed to examine the biofilm proteome of *X. ampelinus* using two-dimensional gel electrophoresis, peptide fingerprinting via mass spectrophotometry and Mascot database search. Given the role played by biofilm formation during initial attachment as well as the regulation of genes expressed in the biofilm maturation process, the purpose of this study was, therefore, to identify the differentially expressed proteins during both initial and mature biofilms following the *in vitro* characterization of *X. ampelinus* biofilm formation.

2.2. MATERIALS AND METHODS

2.2.1. Characterisation of *X. ampelinus in vitro* Biofilm Development

All chemicals used to prepare the growth medium were obtained from Merck (Darmstadt, Germany), Oxoid (Hampshire, UK), and Sigma Aldrich (St. Louis, Missouri, USA). *Xylophilus ampelinus* VS20 strain (obtained from the Agricultural Research Council – Plant Protection Research Institute (ARC-PPRI) culture collection) was revived from a glycerol stock stored at -70 °C by culturing onto YPGA (7 g/L yeast extract powder, 7 g/L Bacto peptone, 7 g/L glucose and 14 g/L bacteriological agar) plates and incubated at 28 °C. After seven days, the bacteria were transferred onto fresh YPGA plates and incubated for four more days.

To observe the development and maturation of a *X. ampelinus* biofilm, a four-day-old cell culture growing on YPGA plates was transferred to 10 ml YPG broth in 50 ml polypropylene conical tubes (SPL Life Sciences, Gyeonggi-do, Korea) to a starting concentration of $1x10^8$ cfu/ml. A sterile glass slide coverslip was inserted inside the tube containing 10 ml bacterial suspension. The tubes were incubated at 28 °C with shaking at 100 rpm. The glass slide coverslips were removed after three, five, seven, ten, and fifteen days, rinsed by dipping into a sterile medium and stained with 0.1% crystal violet solution (Kimix, Cape Town, South Africa). The excess stain was removed by washing the coverslips three times with distilled water after which the coverslips were left to air-dry. A Nikon Eclipse 50*i* light microscope using a 100x magnification and the NIS-Elements software v4.30.01 (Nikon Instruments Inc, USA) was used to view and photograph the biofilms formed on coverslips.

To analyse and quantify the biofilms formed inside glass test tubes, a four-day-old *X. ampelinus* cell culture growing on YPGA plates was transferred to 5 ml YPG broth in 16x150 mm test tubes (Lasec, Cape Town, South Africa) to a starting concentration of $1x10^8$ cfu/ml. The tubes were incubated at 28 °C with shaking at 100 rpm. Biofilm development was assayed three, five, seven, ten, and fifteen days post-inoculation. The tubes were washed three times with water to remove non-adherent cells and were stained with 5.5 ml of 0.1% crystal violet for approximately fifteen minutes. The tubes were rinsed with distilled water, air-dried, and the biofilm rings were photographed. To quantify the biofilm inside the tube, the biofilm was solubilized by adding 5.5 ml of 95% ethanol and the absorbance measured at a wavelength of 600 nm. For statistical analysis

the quantitative assay was analyzed using the GLM procedure for mean comparison in conjunction with analysis of variance using SAS software version version 9.4 (SAS Institute Inc., Cary, USA).

2.2.2. Planktonic and Biofilm Growth Conditions

To obtain sufficient growth for proteomic analysis, a four-day-old *X. ampelinus* VS20 cell culture growing on YPGA was transferred to 300 ml YPG broth in 1000 ml Erlenmeyer flasks to a starting concentration of $1x10^8$ cfu/ml. The cultures were incubated at 28 °C with shaking at 100 rpm.

For the purpose of this study, the initial biofilm at attachment stage (5 days post-inoculation) and mature biofilm (10 days post-inoculation) as determined in section 2.2.1 were examined for differential protein expression against planktonic cells (exponential-growing culture not attached to surfaces) harvested at seven days post-inoculation. At five or ten days post-inoculation, the culture medium was removed and the residual non-biofilm cells were washed off the flasks with 1x phosphate-buffered-saline (PBS) buffer. The initial and mature biofilm samples were then aseptically transferred from culture flasks to microcentrifuge tubes containing 1 ml 1x PBS buffer using a sterile cotton swab. The culture medium containing the planktonic cells was removed seven days post-inoculation. The cells were collected by centrifugation at 8000 rpm for one minute and the cell pellet was resuspended in 1 ml of 1x PBS buffer. The experiment was repeated three times to obtain independent samples for each harvest point.

2.2.3. Protein Extraction

All the equipment and chemicals used in the proteomic study were obtained from BioRad (Hercules, California, USA) unless otherwise stated. Total protein extract was obtained following the conventional TCA/acetone extraction protocol by Chaturvedi and Kumar (2012) with minor modifications. Following the resuspension of the cell pellets with extraction buffer [10 mM Tris-Cl pH 7.8, containing 20 mM 1,4-dithiothreitol (DTT) (Thermo Fischer Scientific, Waltham, Massachusetts, USA) and 1 mM phenylmethylsulfonyl fluoride (PMSF) (Amresco Inc, Ohio, USA), the cells were sonicated on ice using an Ultrasonic Cleaner (ScienTech, Dehli, India) for three to four minutes until all biofilm cell aggregates were disrupted. The cell debris was removed by centrifugation and the supernatant was treated with 20% trichloroacetic acid (TCA) (Merck) in

acetone (Kimix) to precipitate the proteins in the solution. After one hour of protein precipitation at -20 °C, the protein extracts were washed twice with ice-cold 90% acetone to remove all traces of TCA. The protein pellets were air-dried at room temperature and resuspended in rehydration buffer (7 M urea, 2 M thiourea, 2% 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate (CHAPS), 0.5% IPG (immobilized pH gradient) buffer and 0.3% DTT). Urea, thiourea, and CHAPS were obtained from Sigma Aldrich. Following rehydration, the protein extracts were solubilized by gentle vortexing at room temperature for 10 minutes and were stored at -20 °C.

2.2.4. Protein Quantification

All soluble protein extracts were quantified using a modified Bradford assay (Bradford 1976). A total of nine bovine serum albumin (BSA) (Roche, Basel, Switzerland) protein standards were prepared in triplicate from a 1 mg/ml BSA stock solution in a 96-well flat bottom microtiter plate as indicated in Table 2.1. The protein extracts were also prepared in triplicate by mixing 1 µl of the unknown protein sample with 149 µl of 1x Bradford reagent. After adding Bradford solution to samples, the plate was incubated for five minutes at room temperature. Absorbance was measured at 595 nm on a Milton Roy Spectronic GENESYS 5 Spectrophotometer (Spectronic Analytical Instruments, Leeds, UK) using the 0 mg/ml BSA standard as a blank solution. The standards were used to plot a standard curve from which concentrations of all the unknown protein extract samples were deduced.

2.2.5. One-dimensional (1D) Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The protein extracts from the cells growing at the initial attachment and mature stages of biofilm, alongside the planktonic culture were tested for integrity through 1-dimensional SDS-PAGE. Briefly, 5% stacking (40 % acrylamide/bis-acrylamide stock solution; 0.5 M Tris–HCl, pH 6.8; 10% SDS; 10% ammonium persulfate (APS), 16 mM N,N,N’,N’-tetramethylethylenediamine (TEMED)), and 12% resolving (40% acrylamide/ bis-acrylamide stock solution; 1.5 M Tris–HCl, pH 8.8; 10% SDS; 10% APS, 16 mM TEMED) gels were prepared to separate proteins according

to their molecular weights. Ten micrograms of extracted proteins were mixed with 2 µl of 2x SDS loading dye [60 mM Tris pH 6.8, 2% SDS, 10% glycerol, 200 mM DTT, 0.025% bromophenol blue] and denatured by heating at 95 °C for five minutes. Electrophoresis was carried out in 1x Tris-glycine SDS (TGS) running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3) in a Mini-PROTEAN® 3 electrophoresis Cell using a PowerPac™ Universal Power supply. The run was initiated at 100 V for 30 minutes and then adjusted to 120 V. The electrophoresis was performed until the dye reached the bottom of the gel. Following the run, the gel was stained with Coomassie Brilliant Blue R–250 (CBB) and destained with a destaining solution prepared and used as described in section 2.2.7.

2.2.6. Two-Dimensional (2D) SDS-PAGE

2.2.6.1. *Rehydration of IPG Strips*

To identify individual proteins that are differentially expressed in the planktonic, initial and mature biofilm states, 2-dimensional SDS-PAGE was conducted to separate proteins according to their mass and isoelectric points through electro-focusing. One hundred micrograms of protein sample from three replicates representing each growth phase (planktonic, initial, and mature biofilm) was prepared for electro-focusing by adding 50% DTT and 0.2% ampholyte in rehydration buffer. A total of 125 µl per sample was loaded into ReadyStrip™ IPG strips (7 cm long) of pH range 4-7 by laying of the strip onto the sample loaded into an Immobiline™ Dry Strip Reswelling Tray (GE Healthcare, UK). The samples were incubated overnight (16 hours) at room temperature with a covering layer of 1.5 ml mineral oil (PlusOne DryStrip Cover Fluid; GE Healthcare) to prevent the sample from evaporating.

2.2.6.2. *First Dimensional Isoelectric Focusing (IEF) of IPG Strips*

Electro-focusing, a technique for separating different molecules by differences in their isoelectric point (pI), was performed for all samples. The loaded IPG strips were washed twice with distilled water to remove excess protein and mineral oil following the strip reswelling process (described in Section 2.2.6.1) and the strips were dried to remove water drops. The strips were then placed gel side down on the focusing platform of a PROTEAN® IEF CELL and covered with mineral

oil. Isoelectric focusing was performed in a three-phase stepwise program as follows: 250 V for 15 min, 8000 V for 1 hr followed by a total 12 000 Vhrs at 8000 V.

2.2.6.3. *Equilibration of IPG Strips*

After isoelectrofocusing, IPG strips were washed with distilled water to remove the mineral oil and were equilibrated with gentle agitation at room temperature in SDS-containing buffers to solubilize focused proteins and allow SDS binding prior to 2D SDS-PAGE. The IPG strips were incubated gel side up in equilibration tray channels containing 2 ml equilibration buffer [6 M urea, 2% SDS, 50 mM Tris/HCl, pH 8.8 and 20% glycerol] supplemented with 2% DTT for 15 minutes followed by another 15 minutes in equilibration buffer supplemented with 2.5% iodoacetamide (IOA).

2.2.6.4. *Second Dimensional (2D) SDS-PAGE*

The 2D SDS-PAGE gels were cast on 10.1 cm (width) x 8.3 cm (height) glass plates separated by 1 mm thick spacers using the Mini-PROTEAN® 3 Dodeca ™ Electrophoresis Cell gel casting system. A 12% resolving gel solution was prepared as outlined in section 2.2.5. Following equilibration, the IPG strips were gently rinsed by immersing three times in 1x SDS-PAGE running buffer. The strips were then placed on top of the resolving gel phase and were overlaid with 1 ml of 0.5% molten agarose prepared in 1x SDS-PAGE running buffer containing a trace of bromophenol blue. Electrophoresis was performed at 120 V until the dye reached the bottom of the gel. The gels were stained with Coomassie Brilliant Blue R–250 (CBB) stain and destained as described in the section 2.2.7.

2.2.7. Coomassie Brilliant Blue (CBB) Staining and Destaining of SDS-PAGE Gels

Proteins separated by either 1D or 2D SDS-PAGE were detected using a modified CBB R-250 staining protocol that uses three sequential staining steps. After electrophoresis, the gels were dismounted from the gel plate assembly and immersed firstly in CBB staining solution I [0.025% CBB R-250, 10% glacial acetic acid and 25% propan-2-ol], heated for 1 min in a microwave at maximum power and incubated for 30 min with shaking at room temperature. The CBB staining

solution I was discarded and the staining process was repeated using CBB staining solution II [0.003% CBB R-250, 10% glacial acetic acid and 10% propan-2-ol] and finally CBB staining solution III [0.003% CBB R-250 and 10% glacial acetic acid] for 30 min in each stain as described above. After staining, the gels were immersed in destaining solution (10% acetic acid and 1% glycerol] with shaking at room temperature until the protein bands or protein spots (in 1D or 2D gels, respectively) were visibly distinct against a clear background. Three gel replicates of each of the planktonic state, initial, and mature biofilm were imaged using a Molecular Imager PharosFX Plus System.

2.2.8. Comparative Analysis of 2D SDS-PAGE Gels

Comparative analysis of 2D SDS-PAGE gels within defined experiments was done using the PDQuest^{TM} Advanced 2D Analysis software version 8.0.1 build 055. Three replicates of gel images for each state (planktonic, initial, and mature biofilm) were loaded into the software for analysis. One gel representing each growth stage was set as the reference, and the spots of the other replicates were referenced to it. Prior to differential protein expression analysis across the three biofilm phases, the protein spots were manually edited using the group consensus tool to obtain spot expression consensus in all three replicates per test group (i.e. planktonic, initial, and mature biofilm stage). Differentially expressed protein spots were either qualitative (present/absent spots) or quantitative (showing at least a two-fold expression change).

2.2.9. Mass Spectrophotometry

2.2.9.1. *Protein Spot Selection and Handling*

A single gel representative from each biological treatment (initial biofilm attachment, mature biofilm, and planktonic) was selected for picking of spots. Ninety-nine protein spots of interest showing differential expression across stages were picked manually with 200 µl pipette tips and were transferred to sterile 2 ml sterile microcentrifuge tubes for identification using mass spectrophotometry and database searching.

2.2.9.2. *In-Gel Digestion and Peptide Extraction*

All chemicals used for peptide extraction were obtained from Merck unless otherwise stated. The selected gel spots were destained with 200 µl of 50% acetonitrile/25 mM ammonium bicarbonate with occasional vortexing until clear. Samples were dehydrated with 100 µl acetonitrile (ACN) and desiccated using a Speed Vac SC100 (Lab Equip, Dar es Salaam, Tanzania). Proteins were in-gel digested with 100 ng trypsin (Promega, Madison, WI, USA) dissolved in 25 mM ammonium bicarbonate at 37 °C overnight. Peptides were then extracted with 10 µl TA30 solvent (30:70 [v/v] acetonitrile: 0.1% TFA in water) for one hour at room temperature and stored at 4 °C for further analysis.

2.2.9.3. *Mass Spectrophotometric Analysis*

In-gel trypsinized protein fragments were subjected to mass identification using LC-MS mass spectrophotometry at the National Agricultural Proteomics Research & Service Unit (NAPRSU) of the Biotechnology department at University of the Western Cape, South Africa. MALDI-TOF MS and LIFT MS/MS were performed using the UltrafleXtreme MALDI ToF/ToF system (Bruker Daltonics, Bremen, Germany) with instrument control through Flex control 3.4. Approximately 0.5 µl of each digested protein sample was spotted onto an 800 µm MALDI AnchorChip target plate and allowed to air, followed by addition of 0.5 µl α-Cyano-4-hydroxycinnamic acid (CHCA) matrix (1.4 mg/ml). Peptides were ionized with a 337 nm laser and spectra acquired in reflector positive mode at 28 kV using 500 laser shots per spectrum with a scan range of m/z =700 - 4000. Spectra were internally calibrated using peptide calibration standard II (Bruker Daltonics). This calibration method provided a mass accuracy of 50 ppm across the mass range 700 Da to 4000 Da. Peptide spectra of accumulated 3,000 shots were automatically processed using ProteinScape software (Bruker Daltonics).

2.2.10. Data Analysis

The Mascot algorithm using the Swissprot database on a ProteinScape v3.0 workstation was used to process the spectra produced by the MALDI Tof/Tof system. To identify the differentially expressed proteins, the sequenced peptides were searched against the translated coding sequences

(CDs) of the type strain *Xylophilus ampelinus* strain CECT 7646 (LMG 5856; GenBank accession GCA_003217575.1) obtained from the National Centre for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov/assembly). The search parameters were set as follows: bacterial strains, enzyme-trypsin; missed cleavages-1; fixed modification- carbamidomethyl (C); variable modification- oxidation (M); precursor tolerance-50 ppm; 0.7 Da fragment tolerance. According to Mascot probability analysis, only significant (p<0.05) hits were accepted as positive identification. Individual scores that were greater than 16 indicated identity or extensive homology (p<0.05). Individual ions score is calculated as -10*Log (P), where P is the probability that an observed match is a random event. Functional grouping of the identified proteins was done using the KEGG pathway (https://www.genome.jp/kegg/genes.html) as well as literature sources.

2.2.11. Bioinformatic Analysis of the Differentially Expressed Proteins

The RefSeq accession numbers of the proteins identified in section 2.2.10 were used to obtain the corresponding gene locus tags from NCBI. The gene locus tags were then used to identify and locate the open reading frames (ORFs) encoding these proteins within the genome of *X. ampelinus* CECT 7646 (accession number: GCA_003217575.1) using CLC Genomics Workbench v9.0. Further identification and bioinformatics characterization of gene clusters associated with such proteins was done on CLC Genomics Workbench v9.0 to gain insight on certain pathways required for biofilm formation in *X. ampelinus.* The conserved domains of selected proteins were analyzed and confirmed through SMART EMBL (http://smart.embl-heidelberg.de/).

2.3. RESULTS

2.3.1. Analysis of *X. ampelinus in vitro* Biofilm Formation

To understand the dynamics of biofilm development in *Xylophilus ampelinus*, bacterial growth in a liquid medium was monitored over a period of fifteen days to identify the stages of biofilm formation. By examining biofilm formation on glass slide coverslips placed inside 50 ml polypropylene tubes it was apparent that *X. ampelinus* cells undergo four stages of biofilm development i.e. attachment, initial biofilm, mature biofilm, and detachment. During the first two to three days of growth in a liquid medium, the single cells attach to surfaces available to them; the attached cells divide and multiply to form visible micro-colonies over the course of four to five days (Figure 2.1). The micro-colonies grow in size to form primary biofilms between day five and seven and the extracellular biofilm matrix components become visible around the attached cells. These cell aggregates mature by the tenth day forming a thicker 3D-like biofilm structure, which disperses by day 15 (Figure 2.1).

In contrast, biofilm formation in glass test tubes occurred at a different rate, with both visual and quantitative analysis showing an increase in biofilm development even at day 15 (Figure 2.2A and B). Quantitative analysis of biofilm formation in glass test tubes showed a significant increase (P=0.04) in the number of cells within the biofilm from day three to day fifteen post-inoculation (Figure 2.2). This could be due to the differences in the levels of agitation experienced at 100 rpm by cells in each culture vessel, as a result of the difference in diameter of the two types of culture vessels. Biofilm dispersal in glass test tubes (Figure 2.2) did not occur between the tenth and the fifteenth day as observed in the wider 50ml tubes.

Since a large quantity of cells in the biofilm state was required for proteomic analysis, *X. ampelinus* was cultured in 1000 ml Erlenmeyer flasks. The biofilm development pattern in Erlenmeyer flasks was similar to that observed on glass slide coverslips placed inside in 50 ml tubes. Therefore, cells in the initial biofilm formation phase were harvested on day five, while the mature biofilm cells in the Erlenmeyer flasks (Figure 2.3) were harvested on the tenth day for the characterization of *X. ampelinus* initial and mature biofilm proteomes.

2.3.2. One-Dimensional (1D) SDS-PAGE Analysis of *X. ampelinus* Planktonic, Initial Biofilm, and Mature Biofilms Total Proteins

Following the quantification of total proteins extracted from *X. ampelinus* planktonic, initial and mature biofilm cultures, 1D SDS-PAGE was performed to confirm equal loading and the integrity of proteins. The protein bands formed on CBB-stained gels across samples from the three stages of biofilm development showed high similarity, which suggests uniform protein loading (Figure 2.4). The bands were intact and without streaking or smears of degraded proteins indicating that, the proteins were of high quality. The distinct differences in the intensity of the protein bands across stages of biofilm formation were observed and are indicated by arrows (Figure 2.4), indicative of possible differential protein expression.

2.3.3. Two-Dimensional (2D) SDS-PAGE Analysis of *X. ampelinus* Planktonic, Initial Biofilm, and Mature Biofilm Total Proteins

Total protein extracts from three biological replicates of each growth stage (planktonic, initial, and mature biofilms) were separated by (2D) SDS-PAGE, not only according to molecular weight but also according to their isoelectric points (pI). The separated protein spots were visible on the gel following a three-step CBB staining procedure. Comparative representation of protein spots from all independent samples is illustrated in Figure 2.5. Using the PDQuest spot analysis software v8.0.1, approximately 1574 protein spots were observed for all growth stages at a level seven sensitivity. Across all growth stages, 254 spots - showing a two-fold change - were considered to be differentially expressed (Figure 2.5). A total of 99 identifiable differentially expressed protein spots with pure visibility on the gel were selected for identification using mass fingerprinting through MALDI Tof/Tof mass spectrophotometry (MS).

2.3.4. Differential Expression of Proteins in Cells Growing in Initial and Mature Biofilms Compared to Cells Growing in Planktonic State

Eighty-two proteins were identified from 59 spots, with eighteen of the spots yielding multiple protein identities ($p<0.05$), possibly because such proteins may have co-migrated as a result of having the same molecular weight and/ or pI as seen in studies of *Xanthomonas* species (Artier et

al. 2018; Zimaro et al. 2013) and also in a *Ralstonia solanacearum* proteomic study (Dahal et al. 2010). It should be noted that the theoretical mass of 20 proteins were not in agreement with that of the experimental protein masses from the 2D-SDS PAGE images. However, these types of observations are common and were also seen in the *Xanthomonas axonopodis* pv. *citri* biofilm study (Zimaro et al. 2013) and in other studies of phytobacteria (Andrade et al. 2008; Smolka et al. 2003; Xu et al. 2013). This was also observed in a general study of the *X. ampelinus* strain CFBP2098 proteome (Sevillano 2014). Generally, these instances are associated with the sequence errors on the genomic databases, mutations and post-translational modifications among other factors (Abbott 1999; Kim et al. 2016). The proteins in 40 spots remain unidentified, most likely due to low concentrations of protein in such spots. The proteins identified in this study were classified according to their functional categories. Seven prime categories were revealed to have been affected during biofilm formation, and these were cellular processes, environmental adaptation, environmental information processing, genetic information processing, membrane transport, metabolism, and hypothetical proteins of unknown functions. When comparing each stage of biofilm formation with the planktonic stage, seven proteins from each stage were suppressed, while 39 proteins were induced during the initial attachment stage, 35 were induced in mature biofilms. The abundance of twenty-five proteins was reduced in each of the initial and mature biofilm stages investigated.

In both stages of biofilm formation, the majority of proteins involved in genetic information processing, environmental adaptation, and environmental information processing were induced in abundance, whereas the majority of proteins involved in metabolism and membrane transport were reduced in abundance (Fig 2.6 and 2.7). The abundance of four proteins with unknown functions was also induced during biofilm formation with an exception of one protein that was reduced in both stages (Table 2.2).

2.3.5. Proteins Exclusively Induced, Reduced, or Suppressed During Initial and Mature Biofilm Stages

Fourteen and nine proteins were exclusively expressed during initial and mature biofilm formation, respectively when compared to the planktonic stage (Table 2.3). These proteins were either induced, reduced or suppressed in the stage where they were exclusively expressed. Five proteins

were induced only in initial biofilms and remained unchanged in mature biofilms while nine were induced only in mature biofilms (Table 2.3). Of the nine proteins induced during the initial biofilm stage, five were associated with translation. Four and five proteins were reduced in initial and mature biofilms, respectively when compared to the planktonic stage. Only 3-hydroxypropanoate dehydrogenase RutE was suppressed in initial biofilms but remained unchanged in mature biofilms. In addition to these exclusively expressed proteins, two other proteins showed differential expression between the two biofilm stages, namely a starvation-inducible DNA-binding protein (PYE73359.1), which was reduced in initial biofilms and induced in mature biofilms and histidinol dehydrogenase HisD (PYE78275.1), which was reduced in initial biofilms and suppressed in mature biofilms.

2.3.6. Proteins Involved in the Regulation of Biofilm Formation

Generally, biofilm formation is regulated by the Rpf-DSF signaling system involving c-di-GMP regulation. In this study, a 260 amino acid protein annotated as enoyl CoA hydratase (PYE76004.1), was induced during both stages of biofilm development. The enoyl CoA hydratase protein has sequence similarity to the *Xanthomonas* and *Xylella* RpfF proteins, responsible for the diffusible signal factor (DSF) synthesis (Figure 2.8). Further investigation of the *X. ampelinus* CECT 7646 genome showed that there are at least four ORFs encoding proteins which have varying degrees of homology (between 26 and 34% similarity at an amino acod level) when compared to the enoyl CoA hydratase protein (PYE76004.1) induced during biofilm formation in this study. Sequence analysis showed that each of these proteins, including PYE76004.1, contains an ECH_1 and 2 conserved domain, similar to those of the RpfF proteins of *X. campestris pv. campestris* strain 8004 and *X. fastidiosa* strain Temecula. The enoyl CoA hydratase gene encoding RpfF protein in *Xylella* and *Xanthomonas* species is usually located within the *rpf* (regulation of pathogenicity factors) gene cluster. Analysis of *X. ampelinus* CECT 7646 genome showed that the enoyl CoA hydratase open reading frame (DFQ15_11737; PYE76004.1) is not located within such an *rpf* cluster in *X. ampelinus.* However, further investigation of the *X. ampelinus* genome revealed the presence of a number of genes encoding proteins which share similarity with *rpf* genes of *Xanthomonas* and *Xylella,* including an aconitase (DFQ15_106104), long-chain CoA synthetase (DFQ15_12220), LytTR family two-component transcriptional regulator (DFQ15_12336), and

PAS domain S-box-containing protein (DFQ15_11311) encoding proteins with similarity to RpfA, RpfB, RpfD, and RpfS, respectively.

In this study, two distinct Per-ARNT-Sim (PAS)-domain containing proteins were identified (accession numbers PYE73423.1 and PYE75855.1), one of which was exclusively induced during the initial biofilm formation stage (PYE73423.1), while the other was induced in both stages of biofilm formation. Sequence analysis identified more of these PAS domain-containing proteins within the *X. ampelinus* genome. Bioinformatic analysis of PYE73423.1 shows that it contains three PAS and three PAC domains at its N-terminus followed by the GGDEF and the EAL domains.

Furthermore, the ATP-dependent Clp protease proteolytic subunit ClpP characterized as a protein-processing enzyme was reduced in both stages of biofilm formation. Analysis of the *X. ampelinus* genome identified four more genes encoding proteins within this protein family viz. *clpA* (DFQ15_12610, *clpB* (DFQ15_13516), *clpS* (DFQ15_1269), *clpX* (DFQ15_10618). Sequence analysis of the ClpB protein showed that this protein contains a GGDEF domain required for the synthesis of c-di-GMP.

Five proteins involved in nucleotide metabolism were differentially expressed and among them was the 5′ nucleotidases. The 5′ nucleotidases constitute a ubiquitous family of enzymes that are responsible for the regulation of nucleotide and nucleoside levels in the cells. The 5′ nucleotidase was induced during the initial biofilm formation stage of *X. ampelinus* and showed no change in abundance in the mature biofilms.

2.3.7. Proteins Associated with Response of Bacteria to Environmental Changes

Four transcriptional regulators (LysR, MerR, RpoA, and FrmR) were differentially expressed in *X. ampelinus* biofilms. The protein abundance of LysR and FrmR family transcriptional regulators was induced in both stages of biofilm formation. While the protein abundance of RpoA was induced in mature biofilms but remained unchanged in initial biofilms, the abundance of MerR was reduced only in initial biofilms and remained unchanged in mature biofilms.

The DNA-directed RNA polymerase subunit alpha (RpoA), which catalyses the transcription of genes by activating many operons controlled by transcription regulators such as OxyR, OmpR, NahR, UhpA, MetR, EnvZ, and CatR was induced in mature biofilms only but remained unchanged in initial biofilms. Two other proteins viz. thioredoxin (PYE74975.1) and ATP-dependent helicase, RecG (PYE74211.1) associated with the OxyR transcriptional regulator were also induced in both stages of biofilm formation.

Three (3)-hydroxypropanoate dehydrogenase, an enzyme that breaks down pyrimidines was suppressed during the initial stage of biofilms but remained unchanged in mature biofilms. furthermore, ADP-ribose pyrophosphatase (ADPRase) involved in the hydrolysis of excess ADP-ribose to produce AMP and D-ribose 5-phosphate was induced only in mature biofilms but remained unchanged in initial biofilms. Several proteins involved in transcription and posttranslational processing of the genetic information were differentially expressed. i.e. the translation elongation factors (Ts and G), 30S ribosomal protein S1, and SSU ribosomal protein S1P involved in protein elongation during the translation process were induced only during the initial biofilm formation stage. A circularly permuted ATP-grasp protein was reduced only in mature biofilms.

Arginyl-tRNA synthetase generally known for its role of aminoacyl-tRNA biosynthesis during the translation process was induced in both stages of biofilm formation. In addition to this role, this protein also generates AMP, which also exists as a cyclic structure known as cAMP used by the cells in intracellular signaling and gene regulation (Green et al. 2014; Pastan and Parlman 1970). Furthermore, tRNA dimethylallyltransferase critical for the biogenesis of tRNA was also induced in both stages of biofilm formation. Apart from its role in tRNA modification, tRNA dimethylallyltransferase is also involved in the regulation of cytoplasmic translation in response to environmental stresses. Bioinformatic analysis of the tRNA dimethylallyltransferase shows that the protein contains the HAMP (Histidine kinases, Adenylyl cyclases, Methyl binding proteins, Phosphatases) domain generally found in association with other domains such as the PAS repeats, the EAL, GGDEF, or the response regulatory domains.

2.3.8. Proteins Contributing to Formation of Major Components of the Biofilm

Two proteins associated with EPS biofilm matrix components were identified in this study. The first being the cellulose 1,4-beta-cellobiosidase enzyme involved in the catalysis of the hydrolysis of (1,4)-beta-D-glucosidic linkages in cellulose and cellotetraose, releasing cellobiose from the non-reducing ends of the chains, was reduced and suppressed, respectively, in the initial and mature biofilm cells (spots 40 and 13). The differences in the expression of this protein between the two spots may be because spot 40 contained a second protein (acetyl/propionyl-CoA carboxylase alpha subunit).

The second protein associated with EPS production is the RNA processing enzyme polyribonucleotide nucleotidyltransferase (pnp), generally known as polynucleotide phosphorylase (PNPase), involved in messenger RNA (mRNA) degradation and gene regulation, whose abundance was reduced during both stages of biofilm development. One of the genes reported to be regulated by pnp in *Escherichia coli* is the poly-N-acetylglucosamine (PNAG), a major polysaccharide component of the biofilm matrix of bacteria.

Furthermore, a flagellar biosynthesis protein FlhA was suppressed in both stages of biofilm formation. Bioinformatic analysis shows that the gene encoding the FlhA protein is located within the gene cluster encoding flagella biosynthesis and function proteins (Figure 2.9). A TadD protein required for Flp pilus assembly was reduced in initial biofilms but remained unchanged in mature biofilms. None of the proteins associated with the type IV pili (T4P) associated with surface attachment, twitching motility, and biofilm formation were identified in this study.

2.3.9. Proteins with Roles in Adaptation and Virulence

A cell division protein, FtsA, was induced during both stages of biofilm formation. In *X. ampelinus*, *ftsA* is located within the gene cluster encoding cell division and peptidoglycan synthesis proteins (Figure 2.9). In this study, as for cell division protein, FtsA, a cell shape determination protein CcmA was induced in both stages of biofilm formation. Additionally, aspartate-semialdehyde dehydrogenase (PYE79226.1) and aspartate kinase (PYE79356.1) enzymes synthesizing the amino acid, diaminopimelic acid, a derivative of lysine required for cell wall biosynthesis were also induced in both stages of biofilm formation. A T4SS protein

VirB6/TrBL was induced in both stages of biofilm formation. The *X. ampelinus* gene, *virB6/trbL*, was localized within a T4SS gene cluster encoding the proteins required for protein secretion and conjugation (Figure 2.9). Furthermore, an avirulence (*avr*) protein was induced during both the stages of biofilm formation. The sequence analysis of this protein showed that it carries an AvrB_AvrC protein family domain associated with hypersensitive response (HR) in plants. The ATP-dependent helicase (RecG) associated with the genes involved in cell redox homeostasis was also induced in both stages of biofilm formation by *X. ampelinus*. Apart from the induction of the thioredoxin protein, 1-Cys peroxiredoxin protein expressing the peroxidase activity that protects bacteria from H_2O_2 and is implicated in the pathogenicity of *Pseudomonas aeruginosa* (Kaihami et al. 2014) was induced in both stages of biofilm formation.

2.3.10. Proteins Required for Metabolism and Regulation

The abundance of the majority of proteins involved in metabolic processes such as amino acid, carbohydrate, energy, and microbial metabolism in the diverse environment was reduced and in few instances suppressed. Most proteins involved in amino acid metabolism were reduced in *X. ampelinus* biofilms.

SucC, one of the major TCA cycle enzymes was exclusively induced in mature biofilms and remained unchanged during the initial biofilm stage, while homodimeric fumarase class I also involved in the TCA cycle was reduced in mature biofilms but remained unchanged in initial biofilms. In *X. ampelinus* biofilms, three enzymes involved in gluconeogenesis (enolase, phosphoenoylpyruvate synthase, and fructose-bisphosphate aldolase) were reduced during both stages of biofilm formation with an exception of the D-fructose 1,6-bisphosphatase enzyme that was induced in initial biofilms but remained unchanged in mature biofilms. Phosphomannomutase and dihydrolipoyl dehydrogenase also involved in the glycolysis and gluconeogenesis pathways were reduced during both stages of biofilm formation. Although gluconeogenesis and glycolysis pathways are two opposing reactions, none of the glycolysis pathway enzymes were differentially expressed in *X. ampelinus* biofilms with an exception of enolase involved in the two pathways. In addition to these, another enzyme, enamidase, involved in the second step of the sub-pathway that synthesizes propanoate and pyruvate from 6-hydroxynicotinate was suppressed during both stages of biofilm formation. Two enzymes directly involved in energy metabolism (ATP synthase F1

sub-complex alpha subunit and ATP synthase subunit beta) were both reduced during initial biofilm formation. ATP synthase subunit beta was also reduced in mature biofilms while ATP synthase F1 sub-complex alpha subunit remained unchanged.

2.4. DISCUSSION

The biofilm formation process involves several developmental stages; including attachment, initial biofilm, mature biofilm, and dispersal or detachment. Using the YPG liquid medium, the times required to reach these stages of biofilm development were identified for *X. ampelinus* and were designated to occur at day 3, 5 and 10, while the dispersal process begins shortly after ten days post-inoculation. The observed, visual and quantitative differences between each stage and planktonic cultures show that there are physiological changes taking place. To study these changes, an *in vitro* protocol aimed at analysing the biofilm proteome of *X. ampelinus* during the initial and maturation stages of biofilm formation was established. Two-dimensional SDS-PAGE coupled with mass-spectrophotometry was used to identify, select, fingerprint, and characterize the peptides showing differential expression when the cells form biofilms. Because biofilm formation empowers the establishment of the pathogen in its host, studying the proteins critical for this function provides insight into the major pathways that may be involved in the pathogenicity and virulence of *X. ampelinus*. Different protein and gene expression patterns observed during biofilm formation have been described for plant pathogens such as *X. fastidiosa* and *X. axonopodis* pv. *citri* (de Souza et al. 2004; Silva et al. 2011; Zimaro et al. 2013). However, in each of these studies, the mature biofilm was characterised. The current study, sought to gain insight into *X. ampelinus* protein expression in both initial and mature biofilm stages and to identify pathways exclusive to either stage of biofilm formation. To this end, differential protein expression was observed in proteins belonging to the major functional categories such as environmental information processing, adaptation, genetic information processing, cellular processes, metabolism, and transport. For the purpose of this study, the proteins were characterized by their general roles in signaling, nucleotide metabolism, transcription, translation and post-translation modification, adaptation and virulence, and metabolism.

i) Signalling

The differential expression of proteins expressed by the cells during their stages of growth investigated in this study (planktonic, initial and mature biofilms) shows that the cells growing in biofilms are different from the free-living planktonic state. These results showing the induction, reduction and suppression of certain proteins in the cells growing under biofilm conditions when compared to the planktonic stage, indicate that there are coordinated changes in the gene expression of the cells as they develop into a mature biofilm. The changes observed in the proteins expressed during the initial and mature biofilms also show that during each stage of biofilm development, the cells undergo behavioural changes to conform to the needs of the community by responding to both environmental and intracellular signals.

Here in this study, several proteins involved in signalling were identified and most were induced under biofilm conditions, indicating that the cells engage in cell-to-cell communication to co-ordinate the behavioural responses beneficial for their survival in the environment. One of such proteins was the enoyl CoA hydratase. Apart from fatty acid metabolism, enoyl-CoA hydratase also plays a major role in signaling. In some bacterial species such as those belonging to the genera *Xanthomonas* and *Xylella*, enoyl-CoA hydratase is designated as *rpfF* belonging to the *rpf* (for the regulation of pathogenicity factors) gene cluster required for synthesis and perception of the diffusible signal factor (DSF) quorum-sensing (QS) signal (Barber et al. 1997; Newman et al. 2004; Ryan et al. 2015). The Rpf-DSF system is required by plant pathogens for adaptation, motility, biofilm formation, and regulation of virulence. The synthesis of virulence factors in the plant pathogens, *X. campestris* pv. *campestris* and *X. fastidiosa*, is controlled by cell-to-cell signaling mediated by the diffusible signal factor DSF (Barber et al. 1997; Wang et al. 2012). The enoyl CoA hydratase enzyme (PYE76004.1) with similarity to the DSF-synthase RpfF proteins (*X. campestris* pv. *campestris* CAA70872.1 and *X. fastidiosa* Temecula1 AAO28287.1) was induced during both initial and mature *X. ampelinus* biofilm stages. The induction of an RpfF-like protein and identification of other genes with similarity to the *rpf* clusters of *X. campestris* pv. *campestris* and *X. fastidiosa* indicate that *X. ampelinus* may require DSF signaling to regulate the expression of genes required for biofilm formation and virulence as seen in *Xanthomonas* and *Xylella* species. Proteomic analysis of *X. axonopodis* pv. *citri* mature biofilms also reported the induction of RpfN, an outer membrane porin that also forms part of the *rpf* gene cluster (Zimaro

et al. 2013). The *rpf* genes in *Xylella* and *Xanthomonas* species regulate several functions such as virulence, motility, biofilm formation, and dispersal (Barber et al. 1997; Ionescu et al. 2013; O'Connell et al. 2013).

The regulation of these pathways depends on the perception of the DSF signal by the *rpf* cluster signal transduction proteins such as the sensor RpfC and the response regulator, RpfG. Recently, An et al (2014) identified RpfS, a second sensor of the DSF signal, which controls a subset of genes distinct from those controlled by the sensor, RpfC, in *X. campestris* pv. *campestris*, and includes genes involved in the type IV secretion system (T4SS) and chemotaxis. An *X. ampelinus* PAS domain S-box protein (PYE76323.1) with 78% sequence similarity to the *X. campestris* pv. *campestris* RpfS protein was identified. The induction of the RpfF-like enoyl CoA hydratase and identification of an RpfS sensor in *X. ampelinus*, therefore, suggests that the Rpf-DSF signaling system is present and may be required for the control of important functions leading to the virulence of *X. ampelinus*. Identification of proteins with similarity to RpfA, RpfB, and RpfD also confirms that *X. ampelinus* genome encodes Rpf proteins required for signaling and virulence. More in-depth bioinformatic as well as functional characterization of *rpf* genes in *X. ampelinus* is required to verify the mechanism of the Rpf-DSF signaling system and its role in pathogenicity.

Generally, quorum-sensing signals are perceived by the cells and thereafter, coupled to intracellular regulatory networks through a secondary messenger cyclic-di-GMP (c-di-GMP) and a global regulator Clp (He and Zhang 2008). Plant pathogens such as *X. fastidiosa*, and some *Xanthomonas* species require c-di-GMP to regulate major functions in the cell, including the transition of planktonic bacterial cells to biofilm state (Andrade et al. 2006; Chatterjee et al. 2010; Cursino et al. 2015; de Souza et al. 2013; He and Zhang 2008; Ionescu et al. 2013; Ryan 2013; Ryan et al. 2007). The level of c-di-GMP is regulated by the specialized domains present in numerous regulatory proteins. Two-component signal transduction systems in bacteria carry the major domains including PAS, GAF, HAMP, GGDEF, EAL, and HD-GYP, all involved in the regulation of diverse bacterial functions (Galperin et al. 2001).

In this study, two distinct Per-ARNT-Sim (PAS)-domain containing proteins were identified (accession numbers PYE73423.1 and PYE75855.1), one of which was only induced during the initial biofilm formation stage (PYE73423.1), while the other was induced in both stages of biofilm formation. PAS sensors detect a wide range of chemical and physical stimuli and regulate the

activity of functionally diverse effector domains. Bacterial PAS/PAC domains, associated with protein-protein interactions, are commonly involved in environmental sensing (Huang et al. 2003; Möglich et al. 2009; Zhulin et al. 1997). Sequence analysis identified more of these PAS domain-containing proteins within the *X. ampelinus* genome, indicating that these domains are widespread and may be involved in the regulation of diverse cellular functions in response to environmental signals, leading to the adaptation of the pathogen.

As mentioned before, intracellular signaling in bacteria involves the secondary messenger c-di-GMP. The metabolism of c-di-GMP involves its synthesis by diguanylate cyclases (DGCs) and its degradation by phosphodiesterases (PDEs). DGCs containing the GGDEF domain synthesize c-di-GMP whereas PDEs containing the EAL domain or HD-GYP domain degrade c-di-GMP, respectively (Römling et al. 2013). Protein sequence analysis of both PAS-domain proteins identified in this study showed that in addition to PAS/PAC domains, the proteins carry both the GGDEF and EAL domains required for the synthesis and degradation of c-di-GMP. According to Galperin et al. (2001), proteins involved in c-di-GMP metabolism contain the N-terminal PAS domains followed by GGDEF domain and an EAL domain. Bioinformatic analysis of PYE73423.1 shows that it contains three PAS and three PAC domains at its N-terminus followed by the GGDEF and the EAL domains. The PAS domains are used by signaling proteins where they function as a signal sensor domain, while the GGDEF domain is responsible for the diguanylate cyclase activity required for the synthesis of c-di-GMP and EAL domains are responsible for the phosphodiesterase activity required for c-di-GMP hydrolysis. In many bacterial species, modulation of c-di-GMP cellular levels regulate the transition of cells from planktonic to biofilm growth and vice versa. The role of c-di-GMP in biofilm formation is explained in the *Xanthomonas* species through functional characterization of the Rpf-DSF system proteins. The analysis of *rpfG* function, a response regulator carrying an HD-GYP domain responsible for the hydrolysis of c-di-GMP, shows that mutations in this gene result in increased EPS production and biofilm formation and as a response to increased c-di-GMP levels in the cells (Dow et al. 2003; Ryan 2013; Slater et al. 2000). In just as much, expression of genes encoding the EAL domain (with PDE activity) in the *rpfG* mutant of *X. campestris* pv. *campestris* restores extracellular enzyme and xanthan synthesis towards wild-type levels and then triggers biofilm dispersal (Ryan et al. 2006; Ryan 2013). These fundamental facts indicate that biofilm formation is dependent on elevated c-di-GMP levels while

biofilm dispersal occurs when the levels of c-di-GMP decrease in the cell. The induction of the PAS-domain protein (PYE73423.1) during only initial biofilm formation indicates that it may be required for the early transitioning of cells from planktonic to mature biofilm growth. In general, regulation of c-di-GMP levels inside the cells is central to how the bacteria respond to both environmental and cellular signaling, which usually involves the regulation of many critical functions including biofilm formation and virulence in bacteria (de Souza et al. 2013; Ha and O′Toole 2015; Lu et al. 2012; Nakhamchik et al. 2008).

In other bacteria such as *P. aeruginosa*, PAS domain-containing proteins are involved in the regulation of biofilm formation and dispersal processes (Petrova and Sauer 2012b). The induction of PAS domain-containing proteins during *X. ampelinus* biofilm development, therefore, suggests that the bacterium responds to QS signals and modulates intracellular signaling networks to produce the desired response required for the adaptation and survival of the pathogen in its environment. In *P. aeruginosa*, the activation of the protein, BdlA - carrying two PAS domains – by the protease, ClpP, as well as elevated levels of c-di-GMP is required for biofilm dispersal (Petrova and Sauer 2012a, 2012b). In our study, the ATP-dependent Clp protease proteolytic subunit ClpP characterized as a protein-processing enzyme was reduced in both stages of biofilm formation. ClpP belongs to the clp protease family required for protein processing function. Analysis of the *X. ampelinus* genome identified four more genes encoding proteins within this protein family viz. *clpA* (DFQ15_12610, *clpB* (DFQ15_13516), *clpS* (DFQ15_1269), *clpX* (DFQ15_10618). The function of ClpP in the formation of biofilms is illustrated for *Staphylooccus aureus* (Liu et al. 2017). In *Staphylococcus aureus* the ATPases ClpX promote biofilm formation while ClpP represses it (Frees et al. 2004). In this study ClpP was reduced during both stages of biofilm indicating that this protein is not required for biofilm formation. Similar results were observed in *Staphylococcus aureus* biofilms where ClpP was reported to inhibit biofilm formation (Liu et al. 2017).

ii) Nucleotide metabolism

Five proteins involved in nucleotide metabolism were detected with only abundance of phosphoribosylformylglycinamidine cyclo-ligase PurM and orotidine-5'-phosphate decarboxylase induced during both stages of biofilm formation. While 5′nucleotidase was exclusively induced during initial biofilms, ADP-ribose pyrophosphatase YjhB was exclusively induced during the

mature biofilm stage. Three (3)-hydroxypropanoate dehydrogenase RutE was exclusively suppressed in initial biofilms. Nucleotide metabolism is a very important process involving the synthesis and break-down of nucleic acids. Biofilm regulatory pathways involve the activity of dedicated nucleotides including cyclic di-nucleotide (3'3'-cGAMP (cyclic [G(3',5')pA(3',5')p], cyclic di-AMP, and cyclic di-GMP) (Römling et al. 2017). Metabolism of these nucleotides and nucleic acids is vital and critically required for the regulation of major cellular processes. The 5′ nucleotidases constitute a ubiquitous family of enzymes that are responsible for the regulation of nucleotide and nucleoside levels in the cells. The role of 5′ nucleotidases extends to their involvement in maintaining cellular functions, metabolism, and signaling (Santos et al. 2013). Concerning signaling, the enzymes with 5′ nucleotidase activity are important for the dephosphorylation/phosphorylation-dependent activation of molecules involved in the cellular signals that stimulate bacterial biofilm formation process (Gao et al. 2015; Santos et al. 2013). Similar to our result, a 5′ nucleotidase protein is also over-expressed by *X. fastidiosa* cells during the initial stages of biofilm formation, suggesting its role in biofilm regulation (Santos et al. 2013). The induction of nucleotide metabolism proteins in *X. ampelinus* biofilms is related to the regulation of cell functions required for biofilm formation and regulation.

The induction of phosphoribosylformylglycinamidine cyclo-ligase (PYE73809.1) and orotidine-5'-phosphate decarboxylase (PYE74964.1) enzymes required for purine and pyrimidine biosynthesis respectively, during both stages of biofilm formation indicates that when growing in biofilms, *X. ampelinus* cells control cellular functions by regulating gene expression. However, the suppression of 3-hydroxypropanoate dehydrogenase enzyme that breaks down pyrimidines, during the initial stage of biofilms (although remained unchanged in mature biofilms), suggests that during the initial stages of biofilm formation the bacterium experiences an elevated level of gene expression to facilitate the proper transition from planktonic growth to biofilms. Another protein in this sub-category was the ADP-ribose pyrophosphatase (ADPRase) involved in the hydrolysis of excess ADP-ribose to produce AMP and D-ribose 5-phosphate. Excess ADP-ribose generally results from DNA damage in the cell (Bürkle 2001). The induction of ADPRase in mature biofilms, therefore, suggests that the cells may have been experiencing DNA damage, which is generally caused by the presence of toxic compounds such as reactive oxygen species. In this study, the DNA damage and repair-associated protein, ATP-dependent DNA helicase, RecG, was induced in both stages of biofilm formation and is discussed under adaptation. The induction

of DNA damage response proteins in *X. ampelinus* biofilms supports the resulting induction of ADPRase in mature biofilms, confirming the possible accumulation of ADP ribose, which the cells regulate through the activity of ADPRases (Gabelli et al. 2002).

iii) Transcription

Bacteria respond to many signals by expressing or suppressing genes necessary for adaptation and virulence. In this study, four transcription factors that control diverse functions were differentially expressed during biofilm development. The LysR family transcriptional regulator (PYE78914.), induced during both stages of biofilm development plays a critical role in the regulation of virulence in response to cell density by the quorum-sensing signaling system as seen in *Ralstonia solanacearum* (Schell 2000; Tans-Kersten et al. 2004). These LysR regulators also regulate extracellular polymer substances (EPS) production, motility, and biofilm maturation in *R. solanacearum*, *X. fastidiosa*, and *Erwinia carotovora* (Harris et al. 1998; Santiago et al. 2015; Schell 2000; Tans-Kersten et al. 2004). Hence, it appears reasonable to conclude that *X. ampelinus* requires the LysR family transcription regulators to regulate important functions relating to the survival of the bacterium in its environment, biofilm development, and possible establishment *in planta*.

Generally, in bacteria, numerous metabolic processes generate the accumulation of formaldehyde (Denby et al. 2016). The transcription factor, FrmR - induced during both stages of biofilm formation in *X. ampelinus* - regulates the expression of *frmAB*, the GSH-dependent pathway for formaldehyde detoxification. When challenged by the presence of a toxic concentration of formaldehyde, bacteria generally respond by inactivating the FrmR repressor, thereby, inducing the genes, *frmA and frmB* encoding FrmA (formaldehyde dehydrogenase) and FrmB (*S*-formylglutathione hydrolase) (Denby et al. 2016). Due to the induction of FrmR in *X. ampelinus*, one can conclude that formaldehyde did not reach toxic levels during *X. ampelinus* biofilm formation

The DNA-directed RNA polymerase subunit alpha (RpoA) was induced only in mature biofilms of *X. ampelinus*. Similar to this result, RpoA was also induced in *Streptococcus pneumoniae* biofilms (Yadav et al. 2012). RpoA catalyses the transcription of genes by activating many operons controlled by transcription regulators such as OxyR, OmpR, NahR, UhpA, MetR, EnvZ, and CatR

that are required for adaptation, cellular, and functional processes (Frisch et al. 2000; Olechnovich and Kadner 1999; Park et al. 2002; Slauch et al. 1991; Tao et al. 1995). Interestingly, two other proteins viz. thioredoxin (PYE74975.1) and ATP-dependent helicase, RecG (PYE74211.1) associated with the OxyR transcriptional regulator were also identified in this study and are discussed under the adaptation and virulence section. The induction of these transcription regulators in the biofilms of *X. ampelinus*, indicates that the cells respond to the changes in their environment by the expression of genes required for adaptation to the changes around them.

iv) Translation and Post-Translational Processing

Previous biofilm analysis studies showed that only a few genes were expressed similarly in biofilm and planktonic cells, and the majority were differentially expressed (Silva et al. 2011). This fact is supported by the induction of many protein metabolism, translation, and post-translation proteins such as ribosomal proteins, initiation and elongation factors under mature biofilm growth conditions in *X. fastidiosa* and *X. axonopodis* pv. *citri* (Zimaro et al. 2013; de Souza et al. 2004). Results of the *X. ampelinus* study, however, showed that protein metabolism and post-translation proteins were induced only during the initial biofilm formation stage but remained unchanged in mature biofilms. This could also be due to post-transcriptional mechanisms governing the switch from planktonic to biofilm-forming state, which involves the c-di-GMP regulation (Martinez and Vadyvaloo 2014). In our study, one of the PAS domain-containing protein (PYE73423.1) was induced only during the initial biofilm stage and remained unchanged in mature biofilms. Since the function of PAS domain proteins is to regulate protein expression, this observation, therefore, indicates that there is a plethora of genes required for initial biofilm development that were expressed during this stage, hence the difference in protein metabolism and post-translational modification protein abundance between the two stages. Among the proteins induced during *X. ampelinus* biofilm formation, was the translation elongation factors (Ts and G) involved in protein elongation during the translation process. Two more proteins (30S ribosomal protein S1 and SSU ribosomal protein S1P) involved in translation were also induced only during the initial biofilm stage. Interestingly the circularly permuted ATP-grasp protein was reduced only in mature biofilms. The role of this protein involves the synthesis of small peptides. The pronounced induction of protein biosynthesis pathways in initial biofilms compared to mature biofilms could

explain the reduction of this protein in mature biofilms, meaning some protein biosynthesis pathways may be inhibited in mature biofilms of *X. ampelinus*. Arginyl-tRNA synthetase generally known for its role of aminoacyl-tRNA biosynthesis during the translation process was induced in both stages of biofilm formation. In addition to this role, this protein also generates AMP, which also exists as a cyclic structure known as cAMP. In the cells, cAMP is involved in intracellular signaling and gene regulation (Green et al. 2014; Pastan and Parlman 1970). The additional roles of arginyl-tRNA synthetase in AMP production, its involvement in cAMP synthesis, and the need for intracellular signaling and gene regulation during biofilm formation may explain why this protein was not only induced in initial biofilms but also in mature biofilms. Furthermore, tRNA dimethylallyltransferase critical for the biogenesis of tRNA was also induced in both stages of biofilm formation. Apart from its role in tRNA modification, tRNA dimethylallyltransferase is also involved in the regulation of cytoplasmic translation in response to environmental stresses indicating that the phenotypic changes undergone by the cells during the process of biofilm formation are mostly due to environmental stimuli, which drives gene expression and function for the cells to adapt to the changes around them. tRNA dimethylallyltransferase contains the HAMP (Histidine kinases, Adenylyl cyclases, Methyl binding proteins, Phosphatases) domain generally found in association with other domains such as the PAS repeats, the EAL, GGDEF, or the response regulatory domains. This result is also supported by the induction of the PAS-domain containing proteins that modulate the signals channelled into the cells by producing the desired response through c-di-GMP modulation.

Formation of functional 3-D structures of proteins generally occurs through the function of molecular chaperones. Chaperones are stress-responsive proteins induced to facilitate protein folding, refolding, and sometimes to prevent protein aggregation in response to harsh conditions (Lund 2001). The stress-response molecular chaperones have also been implicated in the regulation of gene expression in bacterial biofilms (Beloin and Ghigo 2005). Therefore, the induction of the chaperones (DnaK, HtpG, GroEL) in *X. ampelinus* biofilms shows that their functional responses are required for adaptation and regulation of biofilm formation-associated processes. Similar to our results, DnaK and GroEL were also induced in *X. axonopodis* pv. *citri* and *X. fastidiosa* mature biofilms, respectively (Silva et al. 2011; Zimaro et al. 2013). Because the transition from planktonic to biofilm state involves cellular response to environmental and internal

signals, change in genetic expression, and genetic information processing, functional responses of molecular chaperones may be required.

v) Adaptation and Virulence

Bacterial biofilms are complex structures constituting several components including the extracellular polymer substances (EPS) that form the biofilm matrix, adhesins, and motility structures. During the early stages of biofilm formation, motile bacteria swim towards the surfaces using flagella, which facilitate the rotation of bacteria on the surface during the reversible attachment stage (Guttenplan and Kearns 2014). Flagella motilities are terminated shortly following the initial attachment to the surface when the bacteria produce adhesins, such as EPS, required for biofilm maturation. In this study, a flagellar biosynthesis protein FlhA was suppressed in both stages of biofilm development. In bacterial species, FlhA is involved in the biosynthesis of the flagellum and forms part of the flagellar export apparatus (Ghelardi et al. 2002; Yang et al. 2009). In *X. campestris* pv. *campestris*, FlhA is required for the production of flagellin, FliC, and virulence (Yang et al. 2009). Similar to this observation, flagella biosynthesis genes, *fleN,* encoding flagella synthesis regulator and *flgG* encoding flagella basal body rod protein, were reduced in *P. putida* biofilms (Sauer and Camper 2001). Therefore, suppression of FlhA protein expression observed in this study supports the evidence that the flagella synthesis apparatus may be suppressed in *X. ampelinus* biofilms to promote attachment and maturation of the biofilm. Although *X. ampelinus* was reported to have a single polar flagellum, the genetics underlying this fact was never explored. In this study, analysis of the *X. ampelinus* strain CECT 7646 genome shows that the ORF encoding FlhA is situated within a gene cluster encoding flagella biosynthesis and regulation proteins. Regulation of cellular motility functions, including flagella-dependent swimming motility, have been reported in many bacteria and it requires regulation of signaling molecules such as c-di-GMP (Guttenplan and Kearns 2014).

Aside from the flagellum, bacteria also express an appendage responsible for twitching motility and biofilm formation referred to as the type IV pili (T4P). A study analysing the mature biofilm gene expression profile of *X. fastidiosa* identified two T4P genes, *pilA* and *pilC* that were overexpressed under biofilm conditions (de Souza et al. 2004). However, the analysis of the biofilm proteome of the same bacterium by Silva et al. (2011) only showed induction of PilT and

not PilC and PilA that were previously identified by the genomic study. In our study, none of the T4P complex proteins were identified, even though they may have been expressed under the conditions tested. This may be due to the limitations posed by the methods used in this study that only allows the analysis of proteins within the immobilized pH gradient (IPG) strip with a pH range of 4 to 7 compared to the methods using IPG strips with a 3 to 10 pH range or in solution digest procedure. Alternatively, it could be because in the qualitative method used in this study only protein spots showing a 2-fold difference were considered differentially expressed, which eliminates the identification of proteins with minimal change in expression. However, similar to this study, analysis of the *X. axonopodis* pv. *citri* mature biofilm proteome did not show differential expression of the T4P complex proteins (Zimaro et al. 2013).

Cyclic-di-GMP also regulates the switch of bacteria from motile to sessile, allowing the development of biofilms. Generally, the increased c-di-GMP results in a decrease in motility and increased aggregation through the upregulation of EPS biosynthesis-related genes and downregulation of flagellum genes as seen in *P. aeruginosa* (Guttenplan and Kearns 2014; Mann and Wozniak 2015; Starkey et al. 2009). In this study, the cellulose 1,4-beta-cellobiosidase enzyme involved in the catalysis of the hydrolysis of (1,4)-beta-D-glucosidic linkages in cellulose and cellotetraose, releasing cellobiose from the non-reducing ends of the chains, was reduced and suppressed, respectively, in the initial and mature biofilm cells (spots 40 and 13). The differences in the expression of this protein between the two spots may be because spot 40 contained a second protein (acetyl/propionyl-CoA carboxylase alpha subunit). Cellulose is a biofilm matrix component of the EPS required for biofilm maturation and stability (Jaglic et al. 2014; Wolska et al. 2016). Suppression of the cellulose 1,4-beta-cellobiosidase activity indicates that *X. ampelinus* biofilm formation and maturation processes require cellulose production as the component of the biofilm matrix. This also shows that during both initial and mature biofilm stages of *X. ampelinus,* there is an increased requirement for EPS production, which facilitates the maturation of the biofilms. Specifically, cellulose biosynthesis is regulated by c-di-GMP (Guttenplan and Kearns 2014; Ross et al. 1990), and the suppression of the cellulose-degrading enzymes in this study is most likely to be a response to signals for the increased requirement for EPS production to facilitate attachment and biofilm maturation. These findings are supported by those of Caiazza et al. (2007)

and Guttenplan and Kearns (2014), affirming that following attachment to surfaces, bacteria terminate flagella motilities and produce EPS required for biofilm maturation.

Also concerning the EPS production, the RNA processing enzyme polyribonucleotide nucleotidyltransferase (pnp), generally known as polynucleotide phosphorylase (PNPase), involved in messenger RNA (mRNA) degradation was reduced in abundance during both stages of biofilm development. mRNA degradation is one of the methods used by cells to regulate which genes are expressed and forms part of post-transcriptional gene regulation in bacterial biofilms (Chambers and Sauer 2013; Martínez and Vadyvaloo 2014). One of the genes reported to be regulated by pnp in *Escherichia coli* is the poly-N-acetylglucosamine (PNAG). Pnp inhibits the production of PNAG, thus negatively controlling the formation of biofilms by *E. coli* (Carzaniga et al. 2012). PNAG is a major polysaccharide component of the biofilm matrix of bacteria such as *Bacillus subtilis* (Roux et al. 2015). Therefore, reduction of pnp is required primarily to promote RNA stability and subsequently to promote EPS production and bacterial attachment. RNA stability is critical for cell regulatory functions especially in biofilm regulation by RNA-binding proteins and/ or small RNAs (Chambers and Sauer 2013; Martínez and Vadyvaloo 2014). Regulation of bacterial biofilms by RNA-binding proteins has been observed in many bacterial species (Chambers and Sauer 2013; Lu et al. 2012; Martínez and Vadyvaloo 2014). The related result was reported in *X. axonopodis* pv. *citri* biofilm proteome analysis study where polynucleotide phosphorylase (PNPase) was downregulated in mature biofilms also implicated in the negative regulation of cell aggregation and biofilm formation (Zimaro et al. 2013). The reduction of pnp (PYE79394.1) in *X. ampelinus* biofilms indicates that *X. ampelinus* may produce PNAG as one of the major EPS components required for biofilm development and maturation. However, further investigation of the genes encoding proteins involved in EPS production and regulation is essential.

Population growth and mature biofilm formation require cell division and multiplication. Binary division occurs in adherent cells and the daughter cells spread outward and upward from the attachment point to form clusters (Hall-Stoodley and Stoodley 2002; Tolker-Nielsen et al. 2000). In this study, a cell division protein, FtsA, was induced during both stages of biofilm formation. FtsA is involved in the assembly of the Z-ring, the major component of cell division (Lutkenhaus and Addinall 1997). In *X. ampelinus*, *ftsA* is located within the gene cluster encoding cell division

and peptidoglycan synthesis proteins. In bacteria, cell division is associated with cell-shape determination (Jones et al. 2001). Mutations in some of the cell wall synthesis proteins greatly affect the cell shape as seen in *Bacillus subtilis* species (Abhayawardhane and Stewart 1995). In this study, as for cell division protein, FtsA, a cell shape determination protein CcmA was induced in both stages of biofilm formation. Additionally, aspartate-semialdehyde dehydrogenase (PYE79226.1) and aspartate kinase (PYE79356.1) enzymes synthesizing the amino acid, diaminopimelic acid, a derivative of lysine required for cell wall biosynthesis were also induced in both stages of biofilm formation. The amino acid meso-diaminopimelate is one of the key intermediates of peptidoglycan synthesis and it serves to link the glycan backbones in the cell walls of many bacteria, giving them their shape and rigid structure (Wehrmann et al. 1998). Biofilm development and maturation processes which rely on population growth are thus validated in *X. ampelinus* through the induction of proteins associated with cell division, cell wall biosynthesis, and cell shape.

Bacteria form biofilms as a strategy to adapt to a changing environment. The adaptation process is highly regulated and involves a multitude of changes in genetic information in the cell. One of the ways by which bacteria facilitate adaptation is through the transfer of genetic material from one cell to another. Generally, in bacteria, the genetic transfer is facilitated by the type IV secretion system (T4SS). In addition to this role, T4SS also facilitate the translocation of a wide variety of virulence factors into the host cell. In this study, a T4SS protein VirB6/TrBL was induced in both stages of biofilm formation. The VirB6 protein has demonstrated roles in the T-pilus assembly in *Agrobacterium* species and T4SS function in most bacterial species, e.g. *Neisseria gonorrhoeae*, *Helicobacter pylori*, and *Bordetella pertussis* (Mary et al. 2018; Wallden et al. 2010). The genetic transfer is important for the pathogenesis of plant pathogenic bacteria such as *A. tumefaciens* and *X. fastidiosa* (Bhattacharyya et al. 2002; Zhu et al. 2000). A biofilm gene expression study for *X. fastidiosa* also identified six conjugal transfer proteins (TraG, TraE, TrbE, TrbI, TrbF, and TrbN) that were induced in mature biofilms (de Souza et al. 2004). In this study, the *X. ampelinus virB6/trbL* (induced during initial and mature biofilm stages) was localized within a T4SS gene cluster encoding the proteins required for protein secretion and conjugation. Induction of these genes during biofilm formation could indicate that *X. ampelinus* cells in biofilms secrete substances required for adaptation and virulence, although further functional characterization of

the T4SS cluster in *X. ampelinus* is required. Interestingly, VirB6 expression and T4SS secretion in *Ehrlichia chaffeensis* require the c-di-GMP signaling system (Kumagai et al. 2010), meaning that the T4SS is regulated through quorum sensing. In our study, proteins critical for quorum sensing signaling pathways (proteins with similarity to DSF-synthase, RpfF, and PAS-domain containing proteins) were induced indicating the regulation of important functions through quorum sensing in *X. ampelinus*. However, further analysis of the regulation of biofilm formation and virulence in this bacterium is required.

One of the ways in which bacteria promote virulence in plants is through avirulence genes, which represent a subset of virulence factors involved in the mediation of the host-pathogen interaction. An avirulence (*avr*) protein was induced during both the stages of biofilm formation. During host-pathogen interactions, avirulence genes may act as determinants for pathogen survival. Sequence analysis of this protein showed that it carries an AvrB_AvrC protein family domain. Race 0 and Race 1 of *Pseudomonas syringae* pv. *glycinea* AvrB and AvrC family domain proteins induce the hypersensitive reaction (HR) on specific cultivars of soybean (Staskawicz et al 1987). Generally, *avr* genes encode proteins involved in pathogenicity and are characterized by the induction of HR in plants with corresponding resistance genes or disease in plants without these resistance genes (Leach and White 2002). Although a role for *avr* proteins in pathogenesis is evident, more evidence relating to the role of these proteins in biofilm development must be obtained.

Redox homeostasis is essential for the maintenance of many cellular processes including responses to ROS, signaling, protection of protein thiols, oxidation-reduction reactions as well as the removal of xenobiotics. In this study, thioredoxin, a protein required for redox homeostasis was induced during both stages of biofilm formation. Similar to this result, proteomic and genomic characterization of biofilm formation of the plant pathogens *X. fasidiosa* and *X. citri* subsp. *citri* also showed induction of thioredoxins (de Souza et al. 2004; Moreira et al. 2017). Functionally, thioredoxins act as reductases in redox control, protecting the proteins from oxidative aggregation and inactivation (Berndt et al. 2008). They also help the cells cope with various environmental stresses including programmed cell death (Zeller and Klug 2006). Thioredoxins can directly reduce hydrogen peroxide (H_2O_2) one of the reactive oxygen species (ROS) that mediate programmed cell death (Spector et al. 1988). The oxidoreductase activity of thioredoxin proteins can be reduced by thioredoxin reductase. In this study, thioredoxin reductase was induced only in initial biofilms

but there was no significant change in abundance of this protein in mature biofilms. Concerning thioredoxin, thioredoxin reductase induction during the initial stage of biofilm formation suggests that *X. ampelinus* cells in a biofilm require cell redox homeostasis to evade oxidative stress and maintain the cellular functions. The induction of thioredoxin reductases during the initial attachment state suggest the gradual accumulation of ROS and increased oxidation-reduction reactions as the biofilm matures, which may support the need to prevent oxidative stress by upregulating thioredoxin production while maintaining the normal levels of the reductases.

Generally, the genes that respond to H_2O_2 are found within an OxyR operon including the thioredoxins (Storz and Ames 1990; Zheng et al. 2001). The function of the transcriptional regulator (OxyR) requires an ATP-dependent helicase, RecG, in *Pseudomonas* species (Parvatiyar et al. 2000; Yeom et al. 2012). In this study, the ATP-dependent helicase (RecG) was induced in both stages of biofilm formation by *X. ampelinus*. The function of RecG in biofilms may be associated with the genes involved in cell redox homeostasis, although functional characterization of this protein may be required. In bacteria, H_2O_2 is sometimes produced as a growth-inhibitory substance (McLeod and Gordon 1922). Apart from the induction of the thioredoxin protein, 1-Cys peroxiredoxin protein was induced in both stages of biofilm formation, also confirming the possible presence of H_2O_2. The 1-Cys peroxiredoxin expresses peroxidase activity that protects bacteria from H_2O_2 and is implicated in the pathogenicity of *Pseudomonas aeruginosa* (Kaihami et al. 2014).

The ability of *X. ampelinus* to express these redox-associated proteins in biofilms indicate that when growing in biofilms, the bacterium is better equipped to escape oxidative stress than the planktonic cells. This, therefore, shows that biofilm formation by this pathogen promotes survival under harsh environmental conditions and establishment of the bacterium on the host tissues, thereby contributing to its virulence

Bacteria growing in biofilms enter a starvation-induced growth arrest phase due to depleted nutrients within a biofilm arising from failure to diffuse the nutrients into a biofilm and consumption of the available nutrients by the bacteria in the periphery of the biofilm (Nguyen et al. 2011). When bacteria enter this phase, stress responses are induced. One of the benefits of these responses is increased tolerance towards antimicrobials by the cells in a biofilm, a phenomenon required for virulence (Nguyen et al. 2011). In this study, a starvation-inducible DNA-binding

protein involved in stress response to starvation was induced in cells growing in the mature biofilm stage but was reduced during initial biofilm formation. The induction of this protein suggests that as the *X. ampelinus* biofilm matures, the cells enter the starvation-induced growth arrest phase, necessary for adaptation and survival. Nutrient starvation also plays a very important role in biofilm dispersal, the final stage of biofilm development required for cell-cycle progression. In *Pseudomonas putida* biofilms, nutrient starvation triggers c-di-GMP hydrolysis, which subsequently triggers the expression of genes required for biofilm dispersal (Díaz-Salazar et al. 2017). Biofilm dispersal is dependent on c-di-GMP in the cells as seen in many species such as *P. aeruginosa, Salmonella enterica* serovar Typhimurium, and *Escherichia coli* (Simm et al. 2004; Valentini and Filloux 2016). Gjermansen et al. (2010) conducted the study characterizing starvation-induced dispersion in *P. putida* biofilms and discovered that the hydrolysis of intracellular c-di-GMP led to the dispersal of *P. putida* wild-type biofilm. In *Xanthomonas* species, biofilm dispersal is regulated by the DSF-QS signaling pathway and is required for full virulence in plants (Dow et al. 2003). Therefore, the induction of the starvation-inducible DNA-binding protein during the mature biofilms may be the necessary trigger required for biofilm dispersal of *X. ampelinus*, and may be required for virulence in plants although functional characterization of the genes involved is required.

vi) Metabolism

Previous proteomic and genomic studies of mature biofilms formed by *X. fastidiosa* show increased abundance of the majority of proteins involved in the metabolism of amino acids, carbon, co-factors, energy, nucleotides, fatty acids, DNA, RNA, and proteins (de Souza et al. 2004; Silva et al. 2011). In *X. axonopodis* pv. *citri* mature biofilms, the majority of proteins involved in the TCA cycle were induced whereas other proteins involved in secondary, nitrogen, and energy metabolism were mostly reduced (Zimaro et al. 2013).

In this study, the abundance of the majority of proteins involved in metabolic processes such as amino acid, carbohydrate, energy, and microbial metabolism in the diverse environment was reduced and in few instances suppressed. The reduction of most proteins involved in amino acid metabolism was perplexing because of their general use as critical building blocks for proteins as well as their overall roles in cell growth and development. There is, however, increasing evidence

of the disparate roles of L- and D-amino acids in the cells. For example, previously D-amino acids were not regarded to have major functions when compared to L-amino acids, but recently more evidence has emerged showing they are involved in peptidoglycan synthesis, signaling, and general adaptation of the cells (Cava et al. 2011). Furthermore, these D-amino acids have roles in biofilm dispersal (Kolodkin-Gal et al. 2010). Therefore, the reduction of proteins in this category could be due to their involvement in the biosynthesis of D-amino acids. *Xylophilus ampelinus* may have reduced the activity of such enzymes to promote biofilm development. The induction of aspartate-semialdehyde dehydrogenase and aspartate kinase involved in the synthesis of diaminopimelic acid, a derivative of lysine required for cell wall biosynthesis were also induced in both stages of biofilm formation and are discussed in adaptation and virulence section. Thioredoxin reductase, induced only in initial biofilms is also discussed in the adaptation and virulence section.

The tricarboxylic cycle (TCA), also known as the citric acid cycle or Krebs cycle, is the central pathway in cellular metabolism and involves several enzyme activities. In bacteria, the TCA cycle is generally used by aerobic bacteria to release the stored energy through the oxidation of acetyl-CoA derived from carbohydrates, fats, and proteins into ATP and water. In *X. ampelinus*, SucC was only induced in mature biofilms and remained unchanged during the initial biofilm stage. Similar to this result, SucC was also induced in mature biofilms of *X. fastidiosa* (Silva et al. 2011). In this study, homodimeric fumarase class I also involved in the TCA cycle was reduced only in mature biofilms but remained unchanged in initial biofilms. Generally, the function of fumarases is in the reversible conversion of fumarate to malate. However, recently fumarases were reported to have a secondary role in DNA damage response (DDR) (Singer et al. 2017). In *Bacillus subtilis*, fumarase was reported to be induced upon DNA damage and is required for the DDR (Singer et al. 2017). Interestingly, in this study, another protein involved in DNA damage repair, ATP-dependent DNA helicase RecG, was induced during both stages of biofilm formation by *X. ampelinus*. Due to the differential expression of other proteins involved in cell redox homeostasis in *X. ampelinus* biofilms, it was apparent that RecG was induced as a response to the effects of the cell redox state to promote biofilm formation. Therefore, the reduction of fumarase enzyme in mature biofilms examined in this study simply affirms that *X. ampelinus* may respond to redox stress by promoting biofilm formation and that the accumulation of redox-stress responsive

proteins in later stages of biofilm development reduces the expression of DDR-associated proteins. However, further functional characterization of the role of *X. ampelinus* stress-responsive proteins in biofilm development is required.

Carbohydrate metabolism further involves two major processes i.e. glycolysis and gluconeogenesis. Glycolysis is defined as the metabolic pathway that converts glucose into pyruvate whereas gluconeogenesis generates glucose from non-carbohydrate carbon sources. In *X. ampelinus* biofilms, three enzymes involved in gluconeogenesis (enolase, phosphoenoylpyruvate synthase, and fructose-bisphosphate aldolase) were reduced during both stages of biofilm formation with an exception of the D-fructose 1,6-bisphosphatase enzyme that was induced in initial biofilms but remained unchanged in mature biofilms. Phosphomannomutase and dihydrolipoyl dehydrogenase also involved in the glycolysis and gluconeogenesis pathways were reduced during both stages of biofilm formation. Although gluconeogenesis and glycolysis pathways are two opposing reactions, none of the glycolysis pathway enzymes were differentially expressed in *X. ampelinus* biofilms with an exception of enolase involved in the two pathways. In addition to these, another enzyme, enamidase, involved in the second step of the sub-pathway that synthesizes propanoate and pyruvate from 6-hydroxynicotinate was suppressed during both stages of biofilm formation. Pyruvate can be made from glucose through glycolysis, converted back to carbohydrates (such as glucose) via gluconeogenesis. Pyruvate supplies energy to cells through the TCA cycle when oxygen is present. The suppression of enamidase in biofilms formed by *X. ampelinus* supports the results showing the down-regulation of other enzymes required for glycolysis and gluconeogenesis pathways. The ultimate goal for both pathways is to generate the energy required for other cellular functions. Therefore, the reduction of the levels of these enzymes during biofilm formation indicates that *X. ampelinus* cells growing in biofilms enter a resting state reducing most of the metabolic activities required for energy generation. Interestingly, two enzymes directly involved in energy metabolism (ATP synthase F1 sub-complex alpha subunit and ATP synthase subunit beta) were both reduced during initial biofilm formation. ATP synthase subunit beta was also reduced in mature biofilms while ATP synthase F1 sub-complex alpha subunit remained unchanged. Generally, bacteria require energy to regulate and maintain cellular functions including flagella rotations, which drive cell motilities. A rotary motor that utilizes free energy stored in the electrochemical proton gradient across the cytoplasmic membrane to do the

mechanical work drives bacterial flagella. Using *Streptococcus* strain SM197, Meister et al. (1987) showed that the flux of protons coupled to motor rotation is directly proportional to motor speed. As mentioned previously, a flagella biosynthesis protein FlhA was reduced in both stages of *X. ampelinus* biofilm formation. Down-regulation of flagella synthesis genes, associated with the suppression of flagella motilities, which require energy, suggests that reduction in motility reduces the need for bacteria to synthesize energy, which in a way may have contributed to the reduction of energy-generating pathways. Differential expression of proteins involved in metabolism; therefore, reveals that the central metabolism is affected by the sessile mode of growth in *X. ampelinus*.

vii) Hypothetical Proteins with Unknown Functions

Of the six hypothetical proteins identified in this study, four were induced in both stages of biofilms while only one was reduced in both stages. The induction in abundance of these proteins during biofilm formation suggests that their functions are associated with biofilm development and regulation. Therefore, the functional characterization of these proteins could reveal their roles in biofilm formation and possibly pathogenicity of *X. ampelinus*.

2.5. CONCLUSION

Biofilm formation is still one of the very important pathogenicity factors in bacterial species. Although briefly mentioned in a publication in the past, biofilm formation by *X. ampelinus* has not been studied prior to this. Here we identified the four stages of biofilm formation in *X. ampelinus* and the proteins differentially expressed during both the initial and mature biofilm stages when compared to the planktonic state. Most of the proteins identified were induced in abundance during biofilm formation and were classified in the prime categories including genetic information processing, environmental information processing, environmental adaptation, cellular processes, and hypothetical proteins of unknown functions. From the results obtained in this study, it is apparent that the transitioning of cells from planktonic to biofilm growth states requires a change in the expression of genes to produce phenotypes critical for the survival of the cells in their environment. The striking differences in protein expression observed when comparing the two

stages of biofilm formation indicate that during the developmental processes of biofilm formation from attachment to mature biofilms, there is a shift in the gene expression representing the phenotypic and behavioral changes observed. The differences in protein expression observed when comparing the two stages of biofilm formation also show that some pathways or processes are not affected either in initial biofilms and mature biofilms and that some of these pathways may be critical for one stage but not required for the other stage of biofilm formation. This study identified major pathways required for the regulation of biofilm formation such as the proteins associated with the Rpf-DSF signaling system and c-di-GMP metabolism. The observed induction of proteins required for transcription and post-translational modification of genetic material shows that when transitioning to form biofilms, the cells express certain genetic determinants as a response to the signals brought on by the changes in the environment. As a confirmation to this observation, the study also identified some key transcription regulators involved in the regulation of genes associated with vital functions of the cells including virulence. Functional characterization of such regulators may help identify the genes affected and their functions. The study also identified proteins associated with major components of a biofilm such as EPS, flagella, and other systems required for adaptation and virulence. From this study, it was also apparent that the cells derive many protection benefits from forming biofilms to promote their adaptation, survival, and ultimately virulence. The identification of the induced proteins required for cell wall (peptidoglycan) synthesis shows the investment in protection and survival of cells in biofilms. This study also identified several proteins involved in redox homeostasis, showing that the cells growing in biofilms are more than capable to defend themselves against the accumulation of antimicrobial agents by expressing the genes that promote self-preservation from the deadly environmental factors. From the observation of the proteins with metabolic functions, it is apparent that the central metabolism is affected during biofilm formation. Induction of the majority of proteins with unknown functions shows there are more areas to be explored. Characterization of such proteins might identify some major determinants of pathogenicity seeing that they were induced under biofilm conditions. The results obtained in this study set up ground-work for further identification of pathogenicity factors in *X. ampelinus*.

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TABLES

Table 2.1. Preparation of BSA protein standards for protein quantification.

BSA final concentration (mg/ml)	BSA 1mg/ml stock solution (µl)	Bradford reagent (µl)
0*	0	150
0.006	1	149
0.01	2	148
0.02	3	147
0.026	4	146
0.033	5	145
0.04	6	144
0.046	7	143
0.066	10	140

*Blank solution

Table 2.2. Proteins differentially expressed during *X. ampelinus* initial and mature biofilm formation stages

Spot Number	Gene Locus tag	Protein description	Accession	Species	MOWSE Score[a]	Theoretical [b] MW (kDa)/pI	Experimental[c] MW (kDa)/pI	Expression	
								Initial biofilm	Mature biofilm
1. Cellular processes									
1.1. Cell growth									
37	DFQ15_10457	cell division protein FtsA	PYE78865.1	*Xylophilus ampelinus* CECT 7646	79.59	43.4/5.47	44/5.4	induced	induced
74	DFQ15_10773	cell shape determination protein CcmA	PYE78423.1	*Xylophilus ampelinus* CECT 7646	23.86	21/5.37	24/5.3	induced	induced
80	DFQ15_10341	Flp pilus assembly protein TadD	PYE79054.1	*Xylophilus ampelinus* CECT 7646	16.81	60.2/8.04	30/6.5	reduced	no change
1.2. Cell motility									
11	DFQ15_12150	flagellar biosynthesis protein FlhA	PYE75029.1	*Xylophilus ampelinus* CECT 7646	21.28	73.5/6.3	29/5.4	suppressed	suppressed
2. Environmental adaptation									
36	DFQ15_1023	avirulence protein	PYE79275.1	*Xylophilus ampelinus CECT 7646*	23.1	41.3/9.18	23/5.3	induced	induced
93	DFQ15_11760	hypothetical protein (cell surface protein)	PYE76027.1	*Xylophilus ampelinus* CECT 7646	83.07	56.6/5.55	230/5.4	no change	induced
96	DFQ15_11760	hypothetical protein (cell surface protein)	PYE76027.1	*Xylophilus ampelinus* CECT 7646	84.15	56.6/5.55	245/5.3	no change	induced

3. Environmental information processing									
3.1. Bacterial secretion system									
97	DFQ15_13710	type IV secretion system protein VirB6/type IV secretion system protein TrbL	PYE73359.1	*Xylophilus ampelinus CECT 7646*	19.49	56.5/5.33	245/5.2	induced	induced
3.2. Signal transduction									
97	DFQ15_10343	FAD-dependent sensor of blue light	PYE79056.1	*Xylophilus ampelinus* CECT 7646	18.41	15.4/6.29	245/5.2	induced	induced
4. Genetic information processing									
4.1. Chaperones and folding catalysts									
79	DFQ15_12257	thioredoxin Trx	PYE74975.1	*Xylophilus ampelinus* CECT 7646	50.34	33.8/4.66	29/6.6	induced	induced
4.2. Chromosome and associated proteins									
63	DFQ15_104186	starvation-inducible DNA-binding protein	PYE78990.1	*Xylophilus ampelinus* CECT 7646	47.03	22.1/5.61	22/4.9	induced	induced
4.3. Folding, sorting, and degradation									
33	DFQ15_102127	polyribonucleotide nucleotidyltransferase	PYE79394.1	*Xylophilus ampelinus CECT 7646*	54.3	78.4/5.65	75/5.1	reduced	reduced
52	DFQ15_104141	molecular chaperone HtpG	PYE78947.1	*Xylophilus ampelinus* CECT 7646	40.29	74.8/5.1	69/5.8	induced	induced
53	DFQ15_103220	chaperone protein DnaK	PYE79232.1	*Xylophilus ampelinus* CECT 7646	59.58	69.1/5.04	66/6.1	induced	induced

97	DFQ15_1085	chaperonin GroL	PYE78216.1	*Xylophilus ampelinus* CECT 7646	33.4	57.3/5.05	245/5.2	induced	induced
98	DFQ15_104141	molecular chaperone HtpG	PYE78947.1	*Xylophilus ampelinus* CECT 7646	119.53	74.8/5.1	75/5.9	induced	induced
4.4. Protein processing									
3	DFQ15_10617	ATP-dependent Clp protease proteolytic subunit ClpP	PYE78513.1	*Xylophilus ampelinus CECT 7646*	45.25	22.2/5.51	20/4.8	reduced	reduced
72	DFQ15_11517	putative proteasome-type protease	PYE76150.1	*Xylophilus ampelinus* CECT 7646	92.05	30.8/5.25	29/5.7	no change	reduced
4.5. Replication and repair									
46	DFQ15_1282	ATP-dependent DNA helicase RecG	PYE74211.1	*Xylophilus ampelinus* CECT 7646	136.9	42.5/5.3	42/5.6	induced	induced
4.6. Transcription factors									
8	DFQ15_101227	DNA-binding transcriptional MerR regulator	PYE79906.1	*Xylophilus ampelinus CECT 7646*	23.36	17.2/7.09	52/5.1	reduced	no change
66	DFQ15_104165	DNA-directed RNA polymerase subunit alpha RpoA	PYE78970.1	*Xylophilus ampelinus* CECT 7646	124.65	36/5.8	37/4.7	no change	induced
97	DFQ15_104107	LysR family transcriptional regulator	PYE78914.1	*Xylophilus ampelinus* CECT 7646	19.21	35.7/9.4	245/5.2	induced	induced
99	DFQ15_101223	DNA-binding FrmR family transcriptional regulator	PYE79902.1	*Xylophilus ampelinus* CECT 7646	21.59	10/9.61	58/6.1	induced	induced

4.7. Transcription regulation

45	DFQ15_13410	PAS domain S-box-containing protein/diguanylate cyclase (GGDEF)-like protein	PYE73423.1	*Xylophilus ampelinus* CECT 7646	24.45	110.3/6.21	95/5.3	induced	no change
99	DFQ15_11943	diguanylate cyclase/phosphodiesterase with PAS/PAC sensor(s)	PYE75855.1	*Xylophilus ampelinus* CECT 7646	18.68	84.2/5.47	58/6.1	induced	induced

4.8. Transfer RNA biogenesis

35	DFQ15_102293	tRNA dimethylallyltransferase	PYE79558.1	*Xylophilus ampelinus* CECT 7646	23.63	35.6/9.67	27/5.4	induced	induced
60	DFQ15_102293	tRNA dimethylallyltransferase	PYE79558.1	*Xylophilus ampelinus* CECT 7646	23.63	35.6/9.67	41/5.6	induced	induced

4.9. Translation

6	DFQ15_12916	arginyl-tRNA synthetase ArgS	PYE74190.1	*Xylophilus ampelinus CECT 7646*	86.31	61.9/5.74	57/4.7	induced	induced
16	DFQ15_11059	circularly permuted type 2 ATP-grasp protein	PYE78031.1	*Xylophilus ampelinus CECT 7646*	34.75	53.8/5.45	51/5.6	no change	reduced
17	DFQ15_11265	SSU ribosomal protein S1P	PYE77813.1	*Xylophilus ampelinus* CECT 7646	133.29	61.6/5.27	59/5.6	induced	no change
18	DFQ15_11496	Translation elongation factor 2 (EF-2/EF-G) FusA	PYE76311.1	*Xylophilus ampelinus CECT 7646*	65.01	77.2/5.28	72/5.5	induced	no change
24	DFQ15_10532	ATP-binding cassette subfamily F protein 3	PYE78673.1	*Xylophilus ampelinus* CECT 7646	17.14	76.8/5.89	32/4.3	induced	no change

24	DFQ15_11559	elongation factor Ts (tsf)	PYE76191.1	*Xylophilus ampelinus CECT 7646*	95.09	32.6/6.05	32/4.3	induced	no change
25	DFQ15_11559	elongation factor Ts (tsf)	PYE76191.1	*Xylophilus ampelinus* CECT 7646	40.94	32.6/6.05	35/4.7	induced	no change
48	DFQ15_11265	30S ribosomal protein S1	PYE77813.1	*Xylophilus ampelinus* CECT 7646	26.3	61.6/5.27	60/5.6	induced	no change
5. Membrane transport									
23	DFQ15_11144	iron complex outermembrane receptor protein	PYE77900.1	*Xylophilus ampelinus* CECT 7646	19.44	78.2/7.76	43/6.4	reduced	reduced
40	DFQ15_12643	ATP-binding cassette subfamily B multidrug efflux pump	PYE74324.1	*Xylophilus ampelinus CECT 7646*	20.86	67.2/6.69	61/5.2	reduced	reduced
6. Metabolism									
6.1. Amino acid metabolism									
4	DFQ15_103215	3-isopropylmalate dehydrogenase	PYE79227.1	*Xylophilus ampelinus CECT 7646*	16.93	39.3/5.56	41/5.1	suppressed	suppressed
7	DFQ15_10636	dihydroxyacid dehydratase (ilvD)	PYE78530.1	*Xylophilus ampelinus CECT 7646*	100.89	59.3/5.66	56/4.9	reduced	reduced
8	DFQ15_11180	adenosylhomocysteinase AhcY	PYE77936.1	*Xylophilus ampelinus CECT 7646*	180.94	52/5.63	52/5.1	reduced	no change
9	DFQ15_10864	histidinol dehydrogenase HisD	PYE78275.1	*Xylophilus ampelinus CECT 7646*	24.03	49.2/5.68	51/4.8	induced	suppressed
30	DFQ15_11037	argininosuccinate synthase ArgG	PYE78010.1	*Xylophilus ampelinus CECT 7646*	57.62	49.8/5.57	48/4.7	reduced	reduced

41	DFQ15_10384	glutamine synthetase GlnA	PYE79096.1	*Xylophilus ampelinus CECT 7646*	76.96	52/5.44	55/5.3	reduced	reduced
58	DFQ15_103214	aspartate-semialdehyde dehydrogenase	PYE79226.1	*Xylophilus ampelinus* CECT 7646	24.42	40.1/5.44	38/4.5	induced	induced
66	DFQ15_10442	3-deoxy-D-arabinoheptulosonate-7-phosphate synthase	PYE78850.1	*Xylophilus ampelinus* CECT 7646	30.33	40.6/5.95	38/4.7	no change	induced
84	DFQ15_12429	thioredoxin reductase NTRC	PYE74828.1	*Xylophilus ampelinus* CECT 7646	33.63	34.2/5.46	36/5.6	induced	no change
85	DFQ15_10288	aspartate kinase	PYE79356.1	*Xylophilus ampelinus* CECT 7646	56.63	45.6/5.38	15/52	induced	induced
6.2. C-terminal processing peptidases									
72	DFQ15_11861	S41 family C-terminal processing protease	PYE75935.1	*Xylophilus ampelinus CECT 7646*	21.32	50.9/6.91	28/5.7	no change	reduced
6.3. Carbohydrate metabolism									
6.3.1. Ascorbate and aldarate metabolism									
11	DFQ15_103110	5-dehydro-4-deoxyglucarate dehydratase	PYE79122.1	*Xylophilus ampelinus CECT 7646*	63.89	32.5/5.41	29/5.4	suppressed	suppressed
57	DFQ15_10531	2,5-dioxopentanoate dehydrogenase (NAD+) GabD	PYE78672.1	*Xylophilus ampelinus* CECT 7646	139.33	50.5/6.48	50/4.3	no change	reduced
70	DFQ15_10531	2,5-dioxopentanoate dehydrogenase (NAD+)	PYE78672.1	*Xylophilus ampelinus CECT 7646*	58.91	50.5/6.05	51/4.6	reduced	reduced

6.3.2. Citrate cycle (TCA cycle)

57	DFQ15_10625	homodimeric fumarase (class I)	PYE78519.1	*Xylophilus ampelinus* CECT 7646	18.15	55.5/6.11	50/4.3	no change	reduced
100	DFQ15_12113	succinyl-CoA synthetase beta subunit SucC	PYE74992.1	*Xylophilus ampelinus* CECT 7646	93.91	41.4/5.31	55/6.0	no change	induced

6.3.3. Glycolysis and gluconeogenesis

1	DFQ15_11053	D-fructose 1,6-bisphosphatase	PYE78026.1	*Xylophilus ampelinus* CECT 7646	39.73	37.3/5.81	29/4.4	induced	no change
23	DFQ15_102272	enolase	PYE79537.1	*Xylophilus ampelinus* CECT 7646	123.8	45.6/4.75	43/6.4	reduced	reduced
39	DFQ15_10192	phosphomannomutase	PYE79772.1	*Xylophilus ampelinus* CECT 7646	73.17	48.7/5.49	45/5.2	suppressed	suppressed
42	DFQ15_10183	dihydrolipoamide dehydrogenase	PYE79763.1	*Xylophilus ampelinus* CECT 7646	19.29	64.5/5.5	63/5.3	reduced	reduced
54	DFQ15_10397	phosphoenolpyruvate synthase	PYE79109.1	*Xylophilus ampelinus* CECT 7646	43.04	86.6/5.1	80/5.8	reduced	reduced
61	DFQ15_10192	phosphomannomutase	PYE79772.1	*Xylophilus ampelinus* CECT 7646	60.13	48.7/5.49	47/5.4	reduced	reduced
67	DFQ15_12259	fructose-bisphosphate aldolase (FBA)	PYE74977.1	*Xylophilus ampelinus* CECT 7646	56.08	38.1/5.7	39/4.9	reduced	reduced
69	DFQ15_101244	dihydrolipoamide dehydrogenase	PYE79922.1	*Xylophilus ampelinus CECT 7646*	48.3	52.3/6.21	47/4.5	reduced	reduced

6.3.4. Starch and sucrose metabolism									
13	DFQ15_13514	cellulose 1,4-beta-cellobiosidase	PYE73410.1	*Xylophilus ampelinus CECT 7646*	105	62.1/6.6	57/5.4	suppressed	suppressed
40	DFQ15_13514	cellulose 1,4-beta-cellobiosidase CbhA	PYE73410.1	*Xylophilus ampelinus* CECT 7646	43.98	62.1/6.6	57/5.2	reduced	reduced
6.4. Energy Metabolism									
8	DFQ15_13032	ATP synthase F1 subcomplex alpha subunit	PYE73848.1	*Xylophilus ampelinus* CECT 7646	75.22	51.1/5.17	52/5.1	reduced	no change
15	DFQ15_13034	ATP synthase subunit beta	PYE73850.1	*Xylophilus ampelinus* CECT 7646	77.86	51.1/5.3	50/5.7	reduced	reduced
6.5. Fatty acid metabolism									
40	DFQ15_13512	acetyl/propionyl-CoA carboxylase alpha subunit	PYE73408.1	*Xylophilus ampelinus* CECT 7646	44.66	60.7/5.57	57/5.2	reduced	reduced
74	DFQ15_11737	enoyl-CoA hydratase /carnithine racemase	PYE76004.1	*Xylophilus ampelinus* CECT 7646	32.06	26.7/5.57	25/5.3	induced	induced
6.6. Glutathione metabolism									
36	DFQ15_10211	1-Cys peroxiredoxin	PYE79283.1	*Xylophilus ampelinus* CECT 7646	136.31	23.4/5.57	23/5.3	induced	induced
6.7. Metabolism of co-factors and vitamins									
64	DFQ15_1109	pimeloyl-ACP methyl ester carboxylesterase AreS	PYE77985.1	*Xylophilus ampelinus* CECT 7646	131.71	30.1/5.74	25/4.8	reduced	reduced

6.8. Microbial metabolism in diverse environments

6.8.1. Nicotinate and nicotinamide metabolism

5	DFQ15_10789	enamidase	PYE78439.1	*Xylophilus ampelinus* CECT 7646	86.31	61.9/5.74	45/4.5	suppressed suppressed

6.8.2. Nitrogen metabolism

54	DFQ15_10968	nitrilase	PYE78155.1	*Xylophilus ampelinus* CECT 7646	17.8	29.5/6.16	78/5.8	reduced reduced

6.9. Nucleotide metabolism

24	DFQ15_11726	5'-nucleotidase	PYE75993.1	*Xylophilus ampelinus CECT 7646*	21	37.3/6.09	32/4.3	induced no change
71	DFQ15_13117	phosphoribosylformylglycin amidine cyclo-ligase PurM	PYE73809.1	*Xylophilus ampelinus CECT 7646*	23.31	36.3/5.25	34/5.6	induced induced
74	DFQ15_12246	orotidine-5'-phosphate decarboxylase	PYE74964.1	*Xylophilus ampelinus* CECT 7646	30.44	30.4/5.5	25/5.3	induced induced
75	DFQ15_11820	3-hydroxypropanoate dehydrogenase RutE	PYE75894.1	*Xylophilus ampelinus* CECT 7646	104.37	21.2/5.61	22/5.1	suppressed no change
90	DFQ15_12221	ADP-ribose pyrophosphatase YjhB	PYE74939.1	*Xylophilus ampelinus* CECT 7646	20.8	18.3/4.98	220/5.5	no change induced

6.10. Xenobiotics biodegradation

68	DFQ15_12731	N-ethylmaleimide reductase NemA	PYE74273.1	*Xylophilus ampelinus CECT 7646*	36.05	39.6/5.67	40/4.9	induced induced

7. Unknown

35	DFQ15_12539	hypothetical protein	PYE74366.1	*Xylophilus ampelinus* CECT 7646	22.67	14.9/12.06	23/5.4	induced	induced
64	DFQ15_1365	hypothetical protein DUF1738	PYE73377.1	*Xylophilus ampelinus* CECT 7646	20.28	48.5/8.26	27/4.8	reduced	reduced
73	DFQ15_1232	hypothetical protein	PYE74861.1	*Xylophilus ampelinus* CECT 7646	17.98	31.7/5.29	25/5.7	induced	induced
74	DFQ15_108111	hypothetical protein DUF721	PYE78321.1	*Xylophilus ampelinus* CECT 7646	22.93	11.1/11.66	25/5.3	induced	induced
99	DFQ15_11138	hypothetical protein	PYE77894.1	*Xylophilus ampelinus* CECT 7646	18.09	116.6/6.44	58/6.1	induced	induced

[a]Molecular weight search (MOWSE) scores greater than 16 are statistically significant ($P<0.05$)

[b]Theoretical MW (kDa)/pI - peptide masses and isoelectric points generated by MALDI-TOF MS matched with theoretically generated tryptic digests of all expressed protein sequences from the type strain *Xylophilus ampelinus* CECT 7646 genome sequence in the Mascot database

[c]Experimental MW (kDa)/PI - peptide masses and isoelectric points estimated from the 2D gels shown in Figure 2.5.

Table 2.3. Comparative analysis of proteins exclusively induced, reduced, or suppressed during initial and mature biofilm stages.

Proteins Exclusive to Initial Biofilms		**Proteins Exclusive to Mature Biofilm**	
Protein description	**Accession**	**Protein description**	**Accession**
Induced		**Induced**	
PAS domain S-box-containing protein/diguanylate cyclase (GGDEF)-like protein	PYE73423.1	hypothetical protein (cell surface protein)	PYE76027.1
SSU ribosomal protein S1P	PYE77813.1	DNA-directed RNA polymerase subunit alpha RpoA	PYE78970.1
Translation elongation factor 2 (EF-2/EF-G) FusA	PYE76311.1	circularly permuted type 2 ATP-grasp protein	PYE78031.1
ATP-binding cassette subfamily F protein 3	PYE78673.1	succinyl-CoA synthetase beta subunit SucC	PYE74992.1
elongation factor Ts (tsf)	PYE76191.1		
30S ribosomal protein S1	PYE77813.1	**Reduced**	
thioredoxin reductase NTRC	PYE74828.1	putative proteasome-type protease	PYE76150.1
D-fructose 1,6-bisphosphatase	PYE78026.1	3-deoxy-D-arabinoheptulosonate-7-phosphate synthase	PYE78850.1
5'-nucleotidase	PYE75993.1	S41 family C-terminal processing protease	PYE75935.1
		2,5-dioxopentanoate dehydrogenase (NAD+) GabD	PYE78672.1
		homodimeric fumarase (class I)	PYE78519.1
Reduced			
Flp pilus assembly protein TadD	PYE79054.1		
DNA-binding transcriptional MerR regulator	PYE79906.1		
adenosylhomocysteinase AhcY	PYE77936.1		
ATP synthase F1 subcomplex alpha subunit	PYE73848.1		
Suppressed			
3-hydroxypropanoate dehydrogenase RutE	PYE75894.1		

FIGURES

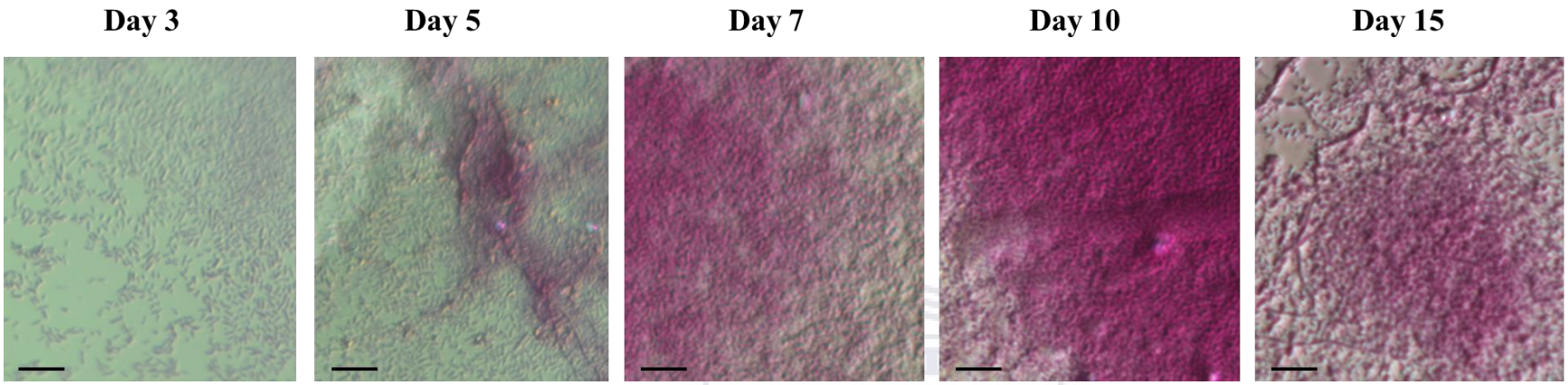


Figure 2.1. Light micrographs showing the stages of *Xylophilus ampelinus* biofilm formation. Development of *X. ampelinus* biofilm on glass slide coverslips over a period of fifteen days. At day 3, initial attachment to the surfaces occur, followed by micro-colony formation at the initial biofilm formation stage; at day seven, the growth of micro-colonies advances to form a primary biofilm; at day ten, the biofilm matures and finally disperses at day fifteen. Biofilm maturation is characterized by the intensity of the crystal violet dye staining the cells attached to the surface of the coverslip while dispersion is signified by less intense dye staining due to the detachment of the cells from the glass surface. Scale bars = 10 µm for all micrographs and were taken under 100x magnification objective.

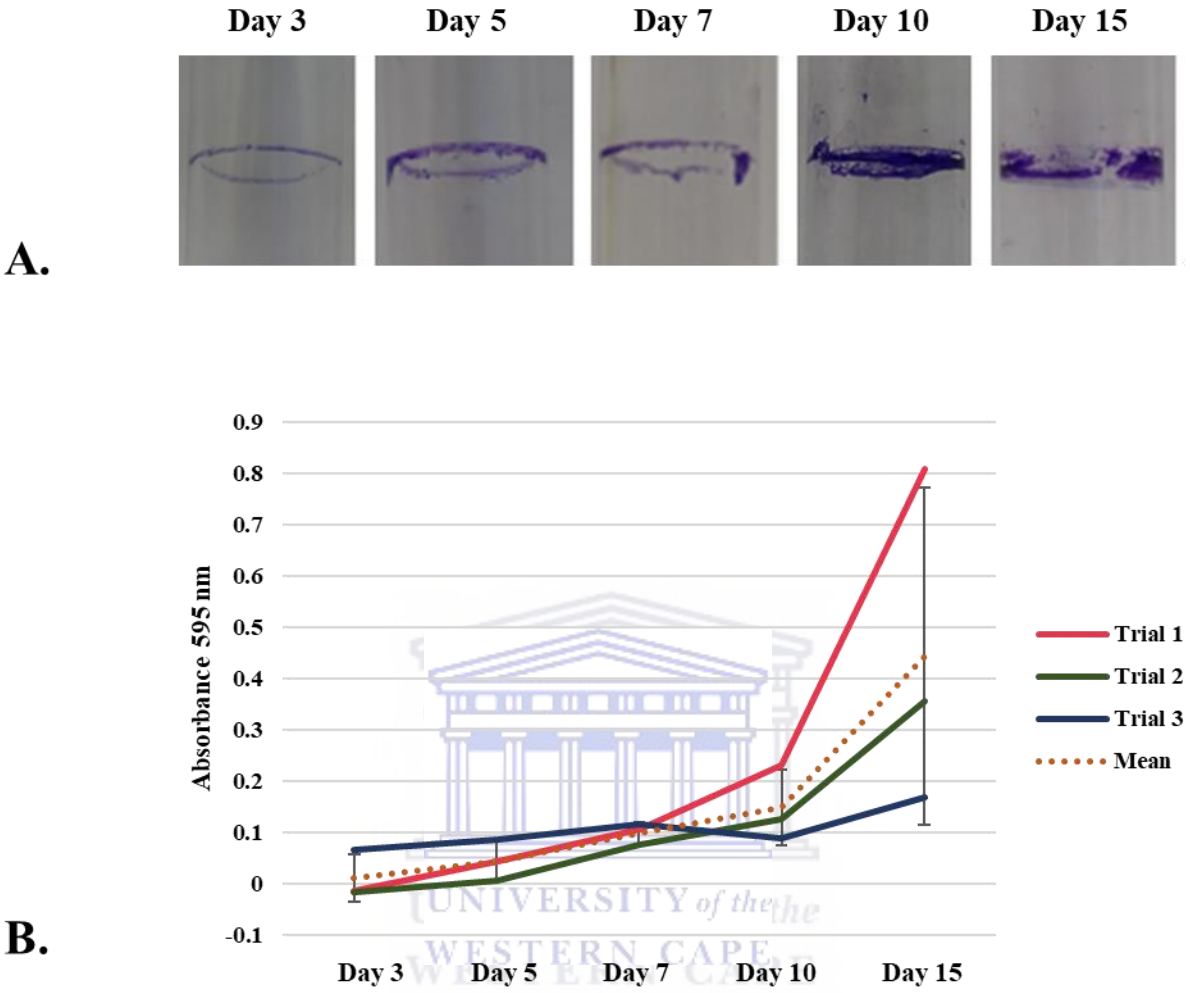


Figure 2.2. Biofilm formation in glass test tubes (A) and biofilm quantification (B) of *Xylophilus ampelinus* over a fifteen and ten day period. The four stages of biofilm development starting from the initial attachment stage at day three, initial biofilm at day five, primary biofilm at day seven, mature biofilm at day ten, and biofilm dispersal stage at day fifteen post-inoculation are shown. The quantification data (B) represent three independent trials with the samples at each time point analysed in triplicate. The average mean and standard error of the three independent trials are shown (P = 0.04).

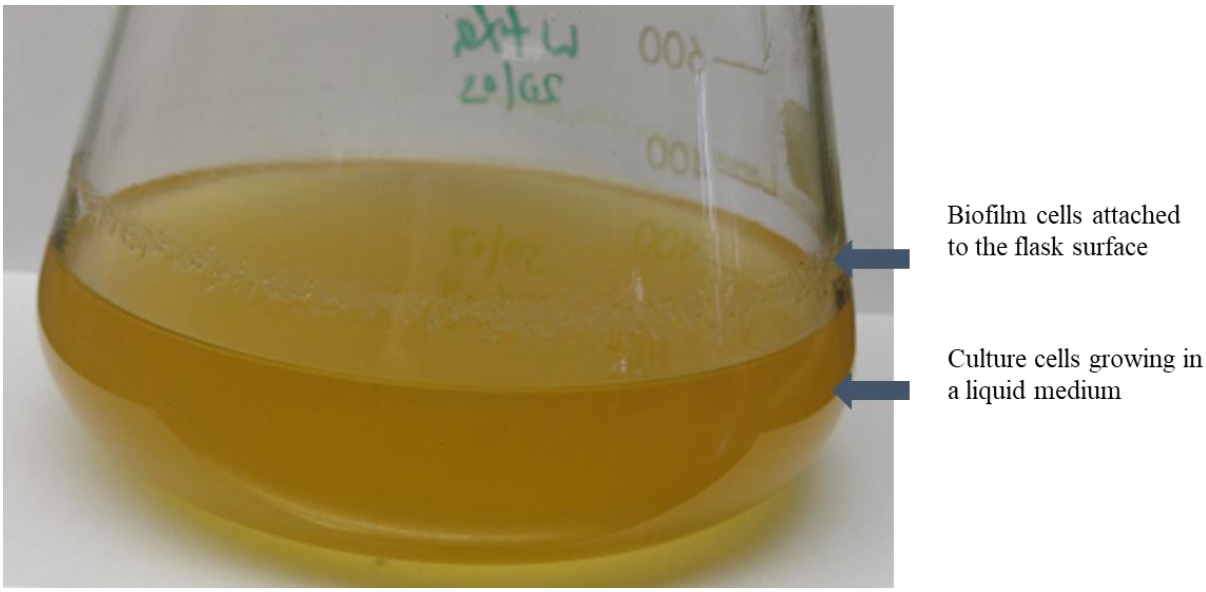


Figure 2.3. ***Xylophilus ampelinus in vitro*** **biofilm and planktonic cultures.** The top arrow indicates the 10-day old mature biofilm cells adherent to the flask, and the bottom arrow indicate the liquid culture containing unattached cells representing a planktonic culture. However, the planktonic cultures used as controls in the proteomic analysis study were obtained by harvesting the cells growing at the exponential phase (7 days post inoculation).

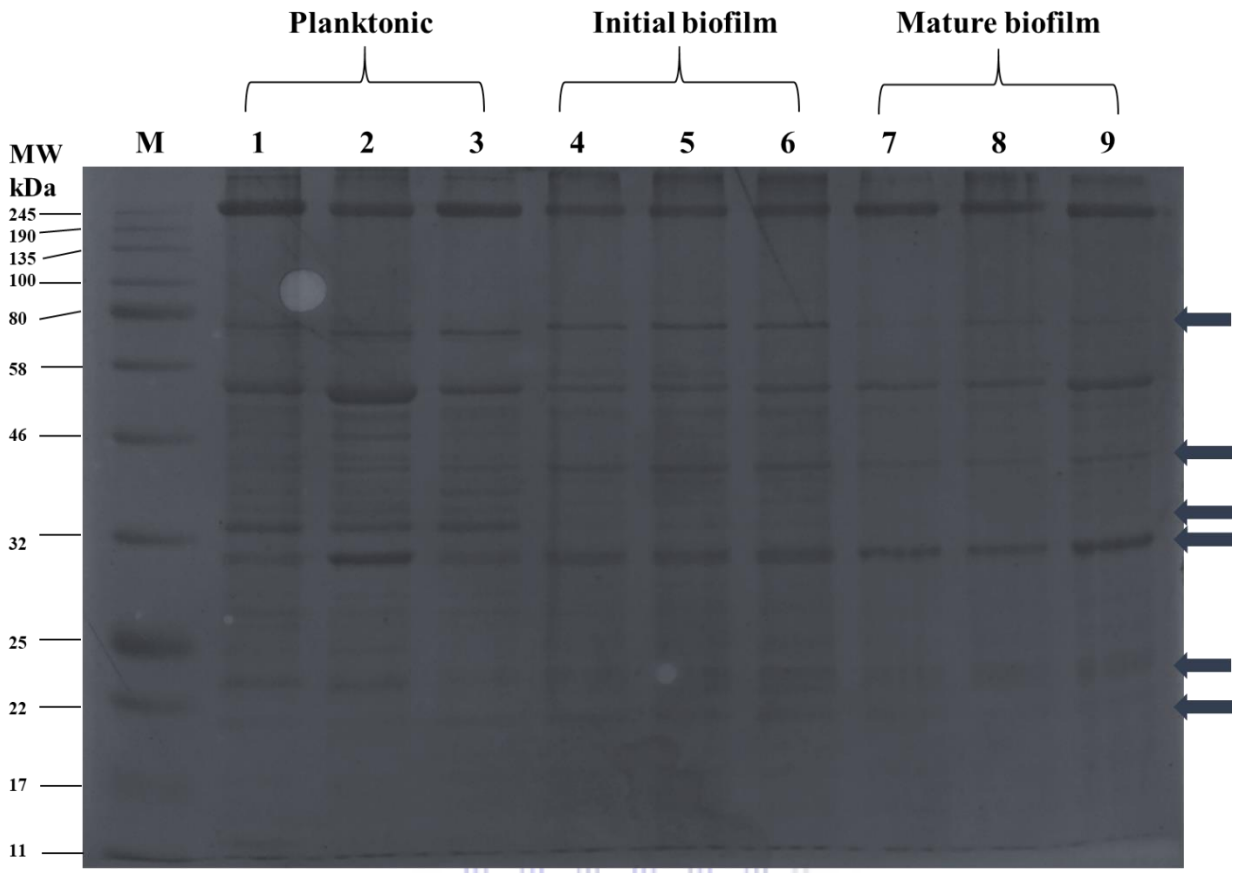


Figure 2.4. Comparative 1D SDS-PAGE analysis of *Xylophilus ampelinus* planktonic, initial biofilm formation, and mature biofilm cell culture proteomes. Lane M- molecular weight marker, Lanes 1, 2, and 3- planktonic protein, Lanes 4, 5, and 6- initial biofilm protein, and Lanes 7, 8, and 9- mature biofilm protein. The arrows indicate differences in the intensity of the protein bands across stages of biofilm formation.

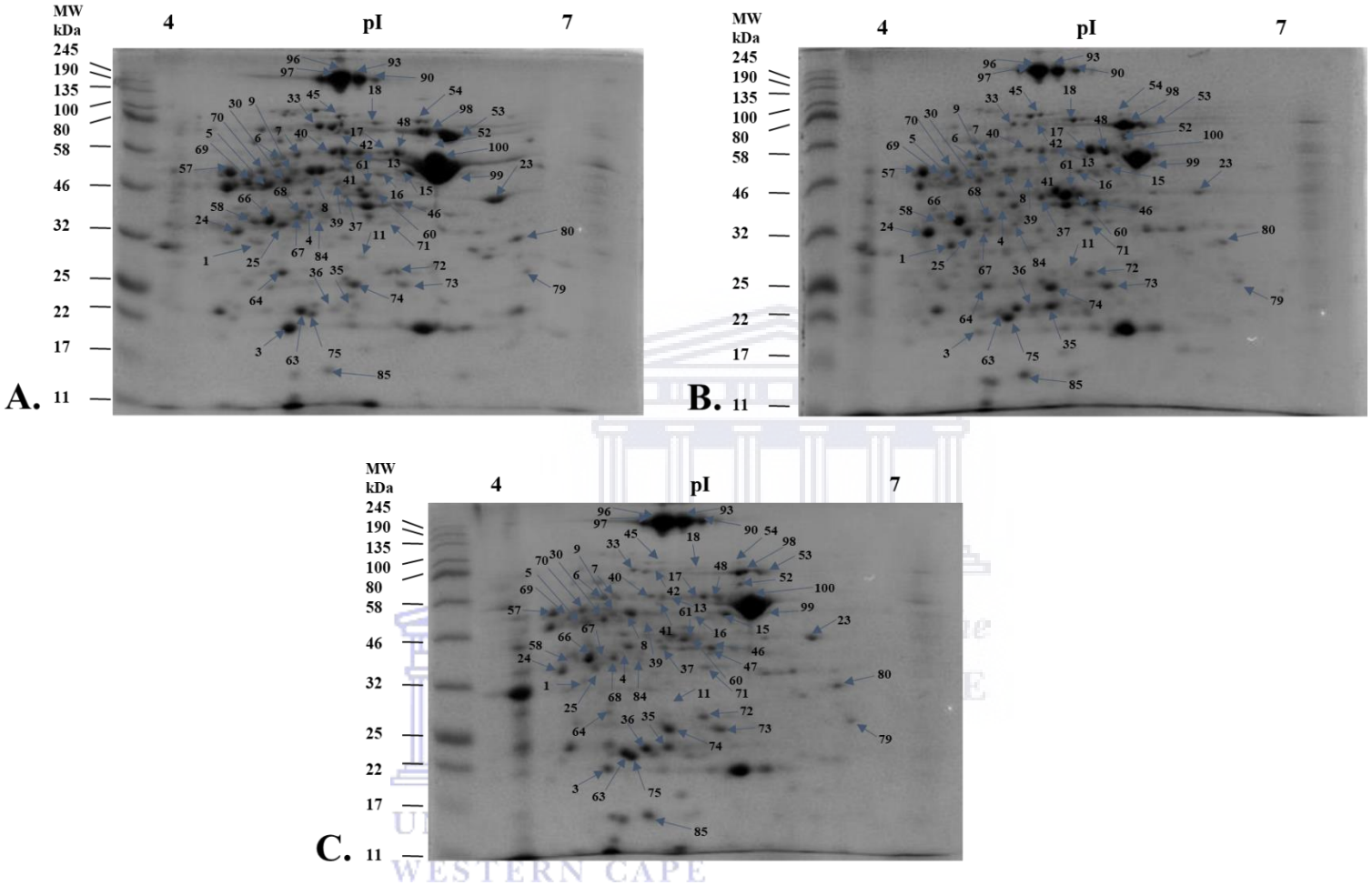


Figure 2.5. Two dimensional SDS-PAGE proteomic profiles for planktonic, initial and mature biofilm growth stages of *Xylophilus ampelinus.* A) Planktonic, B) initial attachment biofilm, and C) mature biofilm. Protein marker appears in a vertical order with molecular weight (kDa) and the IPG strip pH gradient of 4-7. Differential expression is observed through varying intensity of the protein spots on the gels across all stages. The numbers on each gel show only the protein spots of interest as identified by the PDQuest spot analysis software. Each gel is a representative of three replicates of the same stage.

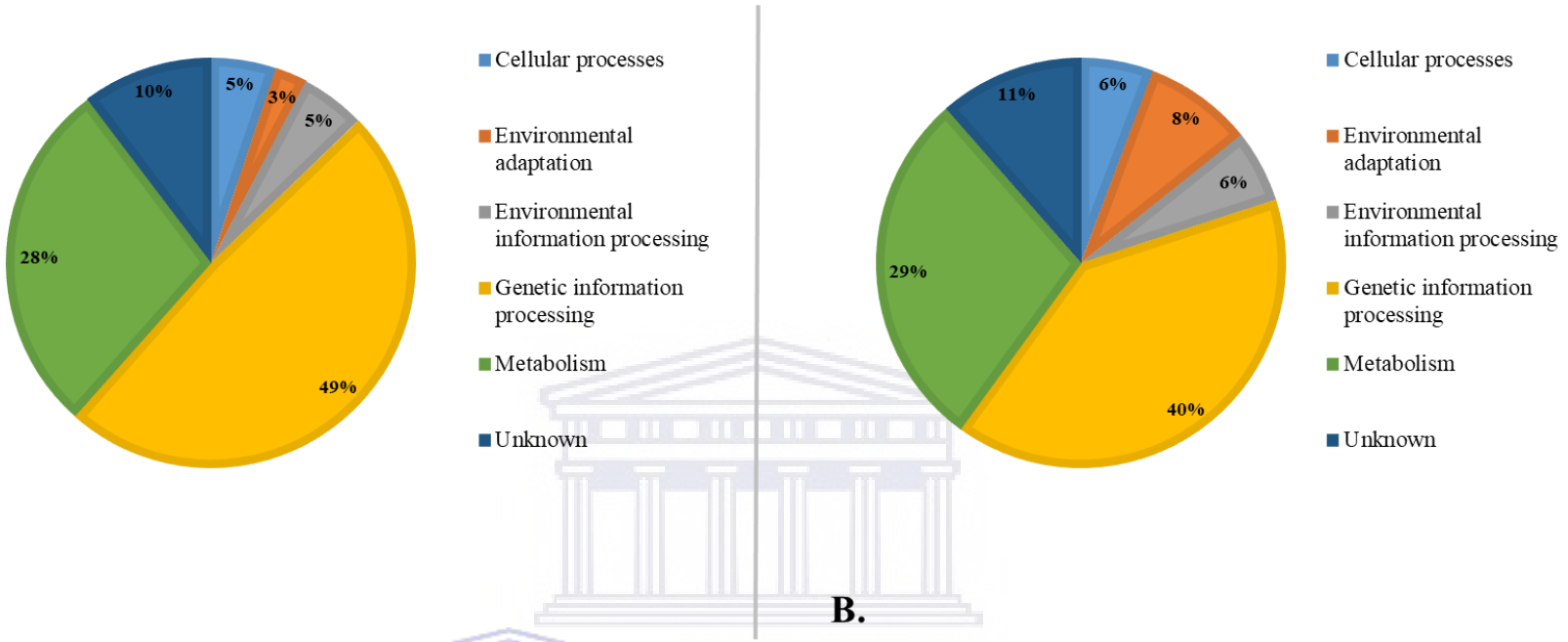


Figure 2.6. Comparative analysis of the biological function categories for the proteins induced in abundance in X*ylophilus ampelinus* cells during (A) initial and (B) mature biofilm formation stages. The identified proteins were grouped according to their biological processes and are expressed in percentage.

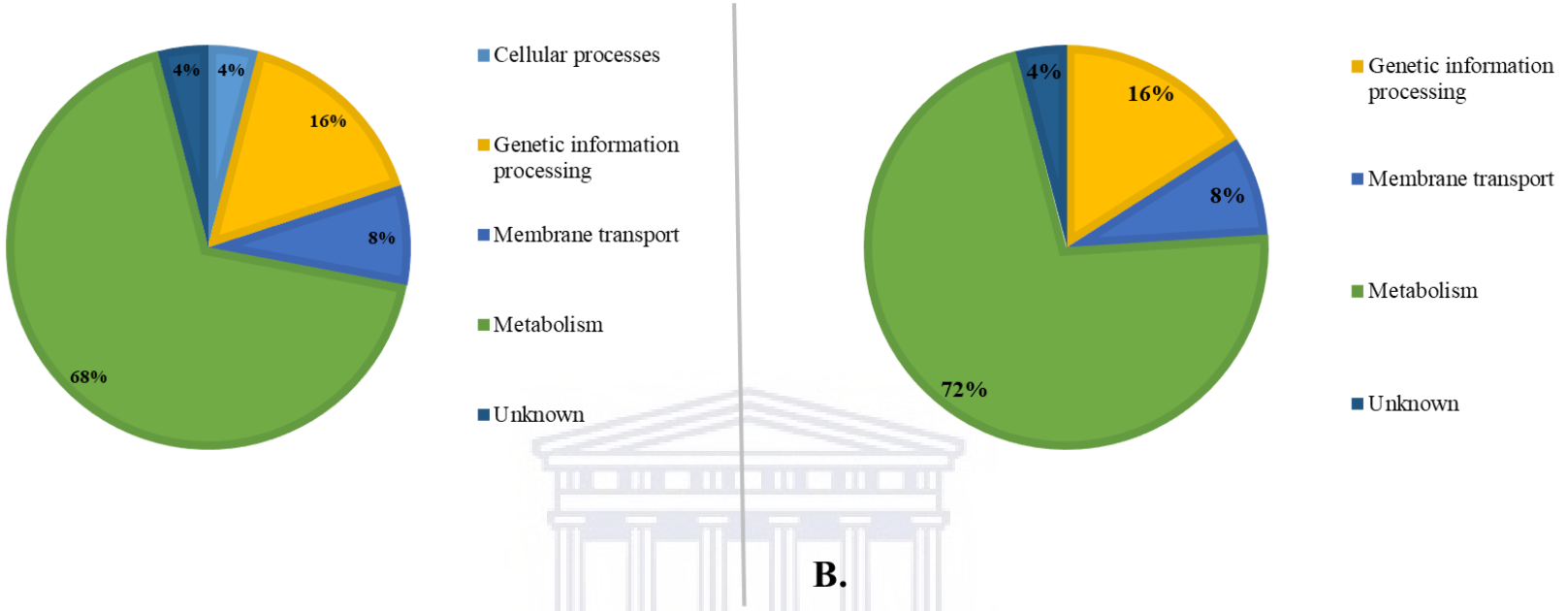


Figure 2.7. Comparative analysis of the biological function categories for the proteins reduced in abundance in *Xylophilus ampelinus* cells during (A) initial and (B) mature biofilm formation stages. The identified proteins were grouped according to their biological processes and are expressed in percentage.

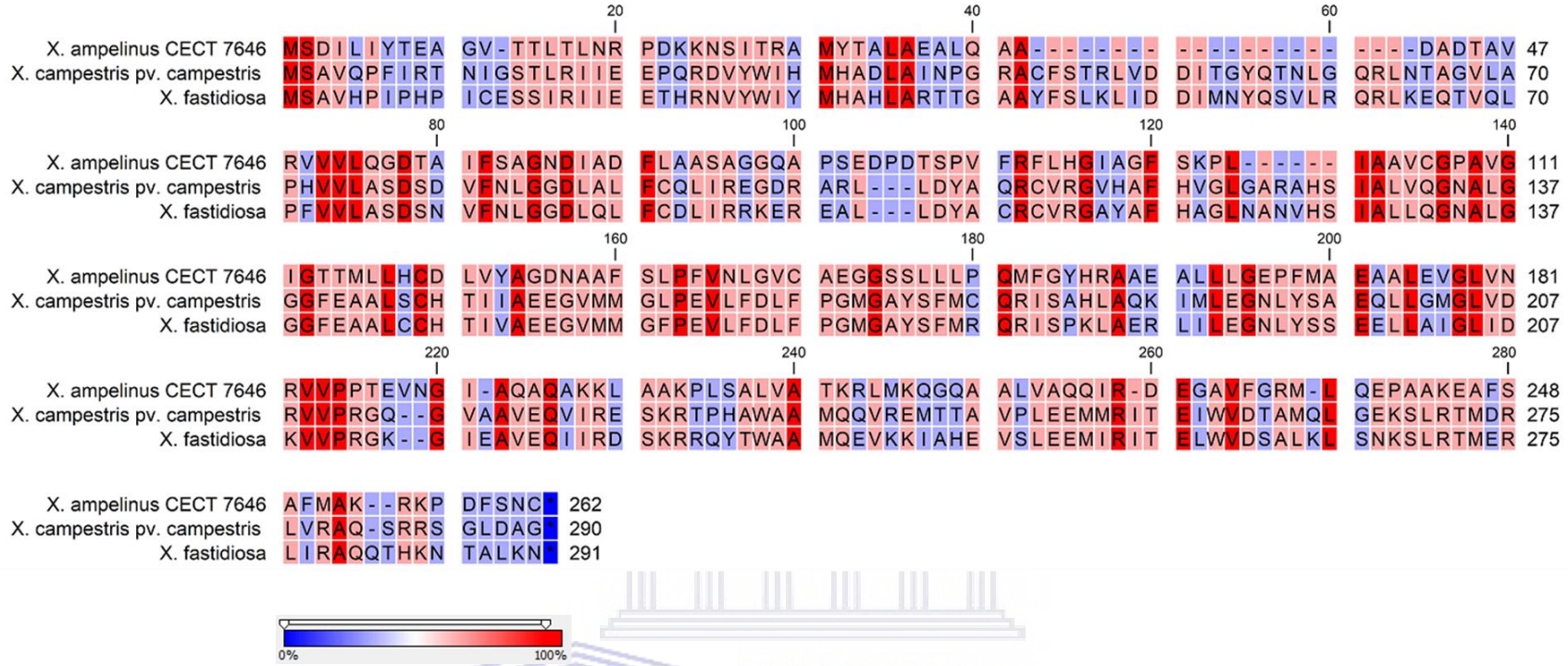
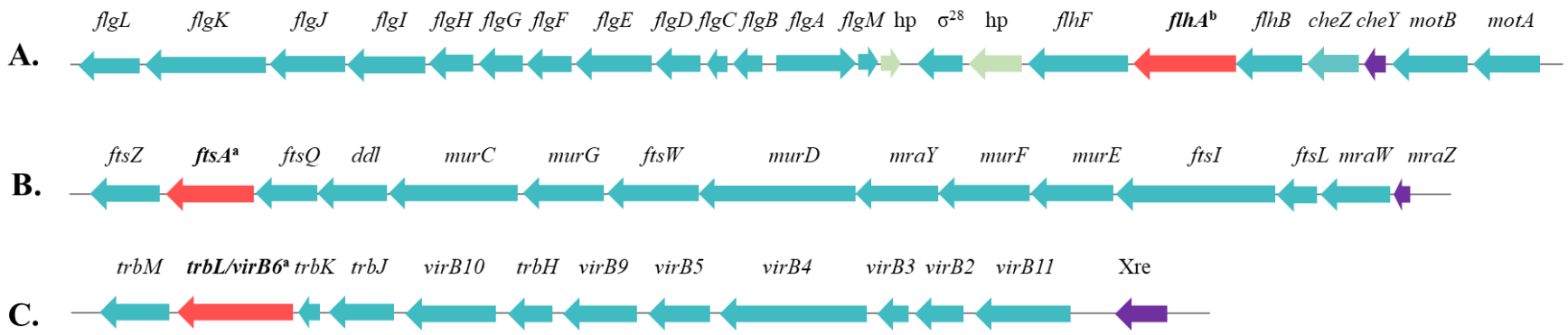


Figure 2.8. Protein sequence alignment of *Xylophilus. ampelinus* CECT 7646 enoyl CoA hydratase (PYE76004.1), induced during initial and mature biofilm stages with *rpfF* protein sequences of *Xylella fastidiosa* Temecula1 (AAO28287.1), and *Xanthomonas campestris* pv. *campestris* str. 8004 (AAY49385.1) generated using CLC Genomics Workbench v9.0. Sequence conservation is illustrated according to the scale provided with dark red colour showing 100% homology.



*[a] – genes encoding the proteins induced during biofilm formation

*[b] – genes encoding the proteins suppressed during biofilm formation

Figure 2.9. Organization of selected *Xylophilus ampelinus* biofilm-associated genes as identified in the genome of strain CECT 7646. The regions illustrated signify A) flagella biosynthesis, B) cell division, and C) type IV secretion system (T4SS) gene clusters. The arrows show the direction of transcription from 5′- 3′. The proteins differentially expressed in biofilms are displayed in orange. The transcriptional regulators are displayed in purple, while the genes encoding the hypothetical proteins are displayed in green.

3. CHAPTER 3: The role type IV pili in biofilm formation and virulence of *Xylophilus ampelinus*

3.1. INTRODUCTION

Xylophilus ampelinus is a Gram-negative, plant pathogen that causes the destructive bacterial blight disease of grapevines. The disease, first identified in Greece (Panagopoulos 1969), has been confirmed to be present in certain vine-growing regions in the Mediterranean, Japan, and South Africa (Bradbury 1986; Dreo et al. 2005; Erasmus et al. 1974; Komatsu and Kondo 2015; Manceau et al. 2005). The bacterium enters the host through open wounds and natural openings and inhabits the xylem vessels of the host plants as biofilms (Grall and Manceau 2003). A biofilm is a consortium of bacterial cells growing on a surface in a self-produced matrix. The biofilm matrix protects the cells from a wide range of environmental factors ranging from extreme temperatures, pH, osmotic stress, desiccation, UV light to anti-microbial agents, and host defenses. Bacteria protected in biofilms are up to 1000 times more resistant to antimicrobial agents compared to their planktonic counterparts existing as free-floating single cells. Biofilm formation is implicated in the pathogenicity of many bacterial species such as *Xylella fastidiosa* and *Xanthomonas* species that cause major plant diseases (Crossman and Dow 2004; Malamud et al. 2013; Marquez et al. 2002; Osiro et al. 2004). The process of biofilm formation is highly regulated in bacteria and it occurs in stages starting from attachment, micro-colony formation, biofilm maturation, and dispersal. Generally, bacteria growing on surfaces and in biofilms engage in surface-associated processes, one being the twitching motility (Mattick 2002; Merz et al. 2000). Twitching motility is the function of the type IV pili assembled inside the cells and expressed outside the bacterial capsule (Mattick 2002). Because of its adhesive nature, bacteria use the T4P to attach to and detach from the surfaces. The actual functioning of T4P involves extension, tethering, and retraction causing the bacteria to translocate in jerky motions resembling the function of a grappling hook (Mattick 2002). This type of motility is employed by bacteria growing on surfaces to translocate across from one surface to another in a colonial fashion (Merz et al. 2000; Turnbull and Whitchurch 2014). T4P are widely distributed among species of archaea and bacteria including diverse members of Cyanobacteria, Firmicutes, and Proteobacteria. Primarily, T4P were shown to be expressed by Gram-negative bacterial species. However, there has been some evidence of T4P

expression by Gram-positive bacterial species (Dubnau 1997; Imam et al. 2011; McKee et al. 2018).

T4P fibres are of variable length and flexibility, ranging between six and nine nanometers in diameter and can be up to four micrometers long. T4P are made up of repeating type IV major pilin subunits and other small auxiliary pilins encoded by the *pil* operon. T4P major pilins, generally secreted as pre-pilins, are relatively small structural proteins with a molecular weight ranging from 7-20 kDa (Giltner et al. 2012). These proteins are comprised of a conserved, hydrophobic α-helical N-terminus that is both a protein-protein interaction domain and a transmembrane domain. T4P pilin proteins secreted by the Sec pathway - a general secretion route which catalyzes the transmembrane translocation of proteins in their unfolded state whereupon they fold into their native structure at the *trans*-side of the membrane - and are subdivided into type IVa and IVb (Natale et al. 2008). Type IVa pre-pilins are characterized by short signal peptides of 5-10 amino acids, while the type IVb pre-pilins have longer signal sequences of 15-30 amino acids. Type IVa pre-pilins are a uniform class found in plant, animal, and human pathogens, whereas the type IVb pre-pilins form a more diverse group found mostly in enteric bacterial species. In the cytoplasm, pre-pilins undergo processing at their N-terminal domains. N-terminal processing of T4P prepilins involves cleavage of the leader peptide sequences at the site between glycine and phenylalanine residues Gly-Phe(Met)Thr-Leu-Ile(Leu)-Glu by the dual function pre-pilin peptidase enzyme PilD, thereby releasing cleaved pilins which then undergo N-methylation by the same peptidase enzyme (Strom et al. 1993). The methylation process refers to the modification of proteins through the addition of a methyl group to the cleavage site and side chains. Apart from the major pilin subunit, some of the proteins required for pilus assembly and function are PilB, PilC, PilD, PilE, PilF, PilM, PilN, PilO, PilP, PilQ, PilT, PilU, PilX, and PilY, while PilR and PilS are the two-component sensor-regulator pair (Alm and Mattick 1997; Mattick 2002). Additionally, PilG, PilH, PilI, PilJ, PilK, and PilL are associated with the chemosensory pathway that regulates the functioning of the system (Alm and Mattick 1997; Mattick 2002; Whitchurch et al. 2004).

During pilus assembly, the mature T4P major pilins encoded by *pilA* are assembled at the base of the pilus fibre on a core inner membrane platform protein encoded by *pilC*. At the base of the pilus structure, there are ATPase proteins functioning in the polymerization and depolymerization of the

pilin subunits. The PilB, PilT and PilU ATPases are the major role players in the extension and retraction of the pilus through the assembly and disassembly of the pilus subunits. The PilB pilus extension protein empowers the assembly by facilitating the addition of the pilin subunits to the growing pilus fibre while the PilT and PilU pilus retraction proteins empower the dissociation of pilin subunits, thereby, facilitating retraction of the pilus. PilT is widely characterized across many species while the function of PilU is not yet fully understood. However, PilU is also required for twitching motility in *Pseudomonas aeruginosa* and in a plant pathogen *Acidovorax citrulli* (Rosenberg et al. 2018; Whitchurch and Mattick 1994). The ATPases are predicted to undergo conformational changes upon ATP hydrolysis, generating a force by converting chemical energy to mechanical energy (Mancl et al. 2016; Satyshur et al. 2007). This generated force thereby facilitates both the translocation of the pilin subunits from the cytoplasmic membrane into pilus during the pilus assembly and the dissociation of subunits back into the membrane during disassembly. The assembled pilus structure is exported to the extracellular surface of the bacterium through type IV pili secretin pores encoded by *pilQ* found in the outer membrane of the cell. At the tip of the pilus is the protein, PilY, that functions as an adhesin required for contact and attachment to the surface.

The role of T4P-mediated twitching motility during biofilm formation is in the formation of micro-colonies (Merz et al. 2000), the stage of biofilm formation that occurs following initial attachment to the surface. During micro-colony formation, the cells move on a surface to form adherent micro-colonies (Merz et al. 2000). T4P-defective bacterial strains are defective in micro-colony formation and ultimately twitching motility and biofilm formation (O'Toole and Kolter 1998). Furthermore, T4P-mediated twitching motility is required for the colonization of upstream vascular regions *in planta* (Meng et al. 2005). The role of T4P in twitching motility, biofilm formation, and virulence of *Pseudomonas syringae*, *Acidovorax avenae*, *Acidovoax citrulli*, *Pantoea ananatis*, and *Ralstonia solanacearum* is also elaborated in their respective systems (Bahar et al. 2009; Kang et al. 2002; Liu et al. 2001; Liu et al. 2012; Rosenberg et al. 2018; Weller-Stuart et al. 2017; Taguchi and Ichinose 2011). Similar attributes of T4P are observed in *Xanthomonas* species, where the role of T4P in the pathogenicity of *X. oryzae* pv. *oryzae* in rice plants and *in vitro* susceptibility to phage infection in *X. citri* subsp. *citri* was also observed (Dunger et al. 2014; Lim et al. 2008).

The process of biofilm formation by *X. ampelinus* in grapevine plants has not been studied, previously. This study sought to investigate the role of the T4P in the formation of biofilms inside the plants and elucidate the potential role in the colonization of of xylem vessels and virulence. To achieve this aim, *in vitro* and *in planta* characterization of the T4P genes (*pilA*, *pilB*, *pilC*, *pilD*, *pilQ*, and *pilU*) required for T4P assembly and function was performed.

3.2. MATERIALS AND METHODS

3.2.1. Bacterial Strains, Plasmids, and Growth Conditions

All chemicals used to prepare the growth medium were obtained from Merck (Darmstadt, Germany), Oxoid (Hampshire, UK), and Sigma Aldrich (St. Louis, Missouri, USA). All bacterial strains used in this study were stored as glycerol stocks at -70 °C. All *X. ampelinus* strains were grown on YPGA (7 g/L yeast extract powder, 7 g/L peptone, 7 g/L glucose and 14 g/L bacteriological agar) plates or in YPG broth at 28 °C for seven to ten days. All *X. ampelinus* T4P mutant and complemented strains were grown on YPGA supplemented with either 10 µg/ml kanamycin or 10 µg/ml kanamycin plus 5 µg/ml gentamycin, respectively. *Escherichia coli* JM109 was grown on Luria-Bertani (LB) plates [10 g/L sodium chloride (NaCl), 10 g/L tryptone, 5 g/L yeast extract powder, 1 g/L glucose, 1 g/L magnesium chloride ($MgCl_2$), and 14 g/L bacteriological agar] or broth at 37 °C for 16 hours. For plasmid propagation in *E. coli*, the LB medium was supplemented with appropriate antibiotics depending on the plasmid maintenance requirements. The characteristics of bacterial strains, plasmids, and antibiotic resistance are listed in Table 3.1.

3.2.2. T4P Gene Sequence Analysis and Gene Annotations

Six major genes encoding the following proteins involved in the assembly and function of the T4P were selected for characterization in this study: i) major pilin subunit (ii) peptidase enzyme (iii) extension and assembly ATPase (iv) retraction and disassembly ATPase (v) inner membrane platform protein, and (vi) an outer membrane secretin.

At the time this part of the research was conducted, the closest related species to *X. ampelinus* with annotated T4P genes was *Acidovorax avenae* subsp. *avenae*; therefore, the T4P protein sequence homologs from this bacterium were used to locate the six open reading frames in the *X. ampelinus* strain VS20 draft genome assembly (unpublished) using the tBLASTn function of CLC Genomics Workbench software v9.0 (Qiagen, Hilden, Germany). The Basic Local Alignment Search Tool (BLAST) search for a retraction and disassembly ATPase using the *A. avenae* subsp. *avenae* protein sequence produced three hits, one carrying a PilT P-loop_NTPase domain while both the other two carried a PulE domain usually found in proteins associated with T4P-mediated twitching motility such as PilU. One of these *pilU*-like genes was situated downstream of the *pilT* gene

encoding the P-loop_NTPase domain-containing protein, and both genes flanked a cyclic AMP (cAMP) CRP protein encoded by *crp/fnr* family of transcriptional regulators known as a c-di-GMP receptor linking cell–cell signaling to virulence gene expression in *Xanthomonas campestris* (Chin et al. 2010). For the purpose of this study, this *pilU* gene, along with *pilA*, *pilB*, *pilC*, *pilD*, and *pilQ* responsible for T4P assembly and function were used to study the role of T4P in twitching motility, biofilm formation, and virulence of *X. ampelinus*. The GenBank accession numbers for the aforementioned six genes are MN582051, MN582046, MN582047, MN582048, MN582049 and MN582050, respectively.

The functional domains of *X. ampelinus* T4P protein sequences were predicted and identified through SMART EMBL online sequence analysis tool (http://smart.embl-heidelberg.de/). To assess and confirm the conservation of T4P sequence across phytobacterial pathogenic species, a pairwise disparity between *X. ampelinus* VS20 protein sequences and T4P orthologues from *Xylophilus ampelinus* CECT 7646 (GCA_003217575.1), *Xylophilus* sp. Leaf220 (GCA_001421705.1), *Acidovorax avenae* subsp. *avenae* (GCA_000176855.2), *Ralstonia solanacearum* (GCA_000009125.1), *Pseudomonas syringae pv. tomato* (GCA_000007805.1), *Xylella fastidiosa* Temecula1 (GCA_000007245.1), and *Xanthomonas citri* pv. *citri* (GCA_000349225.1) was conducted using the NCBI BLASTp function (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The genetic arrangement of the six T4P genes was compared with that of the aforementioned species using CLC Genomics Workbench v9.0.

3.2.3. Primer Design and PCR Amplification

T4P gene deletion mutants were generated using the gene splicing and allelic exchange method in which the gene of interest was replaced by the kanamycin resistance gene. For each T4P gene, PCR overlap extension primers for the sequences approximately 700 bp upstream and downstream of the gene of interest were designed. The primer sequences were designed so they overlap with the kanamycin resistance gene sequence amplified from the EZ-Tn5™ <KAN-2> transposon cassette (Epicentre, Madison, Wisconsin, USA) to replace the gene of interest. The primers to generate the complementation constructs included restriction enzyme sites corresponding to the cloning sites in the vector. All PCR primers listed in Table 3.2 were designed using CLC Genomics Workbench software version 6.5.1 and NCBI Primer-BLAST

(https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and were obtained from Integrated DNA Technologies (Coralville, Iowa, USA).

PCR amplification of DNA segments was done using Phusion High-Fidelity DNA Polymerase (ThermoFisher Scientific, Waltham, Massachusetts, USA) following the manufacturer's instructions. In all PCR reactions, 20 ng of template DNA and 200 nM of each primer was added per 20 µl reaction.

3.2.4. DNA Extraction

Xylophilus ampelinus genomic DNA was isolated using the cetyltrimethylammonium bromide (CTAB) genomic DNA isolation procedure (Ausubel et al. 2003). The air-dried DNA pellet was resuspended in TE buffer (10 mM Tris pH 8.0, 1 mM EDTA) and quantified using the BioDrop DUO spectrophotometer (BioDrop, Cambridge, UK). All plasmids were extracted using the QIAprep® Miniprep kit (Qiagen, Hilden, Germany).

3.2.5. Synthesis by Overlap Extension (SOE) PCR

Synthesis by overlap extension (SOE) PCR is a molecular technique where two or more DNA segments are joined together and extended to form one long segment through PCR. Briefly, the sequences with the overlapping primer sequences complementing one another are fused together to form a double-stranded DNA sequence thereby, synthesizing a new extended strand from the two short strands. SOE PCR was used to synthesize all the constructs for gene knockout mutations. To obtain the SOE recombinant products, two separate PCR master mixes A and B were prepared as described in Table 3.3.

The purpose of PCR reaction A was to join three DNA segments by natural priming. Briefly, the segments were mixed in a tube in the presence of the polymerase enzyme allowing the overlapping sequences on the primer terminals to recognize their complementary strands and fuse together to form a single DNA segment. Phusion Taq was used to hybridize overlapping sequences at the terminals of each fragment. The purpose of PCR reaction B was to amplify the recombinant PCR product. Recombinant PCR product size validation was obtained through electrophoresis in 1x

TBE buffer (8.9 mM Tris, 8.9 mM Boric acid and 2 mM EDTA). DNA bands were visualized under ultraviolet (UV) light.

3.2.6. Construction of Mutant and Complemented *X. ampelinus* Strains

All plasmids were constructed through restriction digest and ligation reactions. FastDigest restriction enzymes, the Rapid DNA Ligation, and CloneJET PCR Cloning kits were obtained from ThermoFisher Scientific and used according to the manufacturer's instructions. The QIAEXII gel extraction kit (Qiagen) was used for all DNA purification from the gel slices according to the manufacturer's instructions. To create *X. ampelinus* T4P gene-knockout mutant strains, in-frame deletions in the *pilA, pilB, pilC, pilD, pilQ,* and *pilU* genes were obtained by a two-step allelic exchange procedure described in (Guzzo et al. 2009). Briefly, the individual T4P gene recombinant PCR product consisting of approximately 700 bp of sequences upstream and downstream of the gene separated by a kanamycin resistance gene was cloned into CloneJET pJET1.2/blunt vector. Following the ligation reaction, all generated plasmids were propagated by transforming *Escherichia coli* JM109 cells made chemically competent according to Sambrook and Russell (2001). Propagated plasmids were isolated using the QIAprep® Miniprep kit (Qiagen) and were validated via restriction enzyme digests. Further validation was done through DNA sequencing of the constructed plasmids at Stellenbosch University Central Analytical Facilities (CAF) DNA Sequencer Unit. All mutant constructs were sequence verified using primers specific for the pJET1.2/blunt cloning vector and the kanamycin cassette, whereas all complementation constructs were verified using *LacZ* operon primers (Table 3.2).

To create *X. ampelinus* T4P deletion mutants, the vector constructs were introduced into electro-competent wild-type *X. ampelinus* cells using a Bio-Rad Gene Pulser™ system coupled with Pulse Controller and Capacitance Extender (Bio-Rad, Hercules, California, USA). Wild-type *X. ampelinus* electro-competent cells were prepared by washing the cells at least six times with ice-cold 15% glycerol following two washes with ice-cold sterile distilled water. From each ligation reaction, 3-7 µl was electroporated into 40 µl cells which were immediately suspended in 900 µl of ice-cold SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose) and allowed to grow at 28 °C for four hours prior to plating onto YPGA supplemented with kanamycin. The kanamycin-resistant colonies were

selected and verified through PCR using the *X. ampelinus* forward primer amplifying the sequence upstream of the native promoter of each gene of interest and the reverse sequence primer of the kanamycin gene. This was to ensure that the gene of interest has been knocked out and has been replaced by the kanamycin gene. To complement these mutant strains, each gene and its upstream promoter sequence were cloned into appropriate restriction sites in the pBBR1MCS5 vector and introduced into their corresponding electro-competent mutant strains as described above. The kanamycin- and gentamicin-resistant colony forming units (CFU) were selected and verified through PCR using the *lacZ* operon primers.

3.2.7. Scanning and Transmission Electron Microscopy (STEM)

All T4P mutants and complemented strains along with wild-type *X. ampelinus* (used as a positive control) were subjected to STEM analysis to confirm the presence or absence of T4P on the bacterial cell surface. The bacterial strains were grown for seven days at 28 °C on YPGA containing 1% agar supplemented with required antibiotics. All STEM micrographs were acquired using the Zeiss MERLIN Field Emission Scanning Electron Microscope at the Electron Microbeam Unit of Stellenbosch University's Central Analytical Facility (CAF). Samples were prepared by placing carbon-coated copper TEM grids mesh-side down onto the edge of colonies solubilized with sterile distilled water for three minutes. The grids were stained with 2% uranyl acetate, washed with deionized sterile water and finally left to air-dry. The Zeiss five-diode Scanning Transmission Electron Detector (Zeiss aSTEM4 A Detector) and Zeiss Smart SEM software were used to generate STEM images. Beam conditions during analysis on the Zeiss MERLIN FE-SEM were 20 kV accelerating voltage, 250 pA probe current, with a working distance of approximately 4 mm. Images were acquired in bright fields mode with the S1 diode activated.

3.2.8. Functional Assays

3.2.8.1. *Twitching Motility Assay*

To investigate the role of T4P in twitching motility, a sterile toothpick was used to pick a colony from a four-day-old culture and transfer it onto YPGA plates containing 1% agar, by stab-

inoculating the agar (Dunger et al. 2014). The process was repeated for all strains and the inoculated plates were incubated for ten days at 28 °C. Twitching zones were observed by studying the colony peripheral edges through viewing under a stereomicroscope (Leica, Wetzlar, Germany) and all colonies were photographed.

3.2.8.2. *Biofilm Formation Assay*

To assess the role of T4P in the formation of biofilms on glass surfaces, 10 ml of 10^8 CFU/ml of all T4P mutants, T4P complemented strains and wild-type *X. ampelinus* were grown in YPG broth supplemented with appropriate antibiotics when necessary. A sterile glass slide coverslip was inserted inside each 50 ml polypropylene conical tube (SPL Life Sciences, Gyeonggi-do, Korea) containing 10 ml bacterial suspension. The tubes were incubated at 28 °C with shaking at 100 rpm. All strains were analysed in triplicate and harvested at fixed time intervals of five and ten days to examine the structure and intensity of *X. ampelinus* biofilms formed on glass surface during the initial and mature biofilm states. The glass slide coverslips were removed after five and ten days post inoculation, rinsed by dipping into a sterile medium, and stained with 0.1% crystal violet solution (Kimix, Cape Town, South Africa). The excess stain was removed by washing the coverslips three times with distilled water, after which the coverslips were left to air-dry. A Nikon Eclipse 50*i* light microscope using a 100x magnification immersion oil objective and the NIS-Elements software v4.30.01. (Nikon Instruments Inc, USA) was used to view and photograph the biofilms formed on coverslips.

3.2.9. *In planta* Assays

To study the role of T4P in biofilm formation and host colonization, one-year-old grapevines of the bacterial blight susceptible cultivar Redglobe, grafted onto the rootstock, Ramsey, was inoculated with mutant and complemented strains. The plants were maintained under glasshouse conditions until the freshly growing shoots had at least ten nodes. Two shoots per plant were wounded by making a 1 cm longitudinal cut between nodes six and eight. Four plants per sample were inoculated by infecting the wounds with approximately 30 µl of $1x10^8$ CFU/ml of the bacterial suspension.

The development of symptoms was monitored and photographed from 25 days-post inoculation until the end of the experiment at twelve weeks post-inoculation. At the harvest point of twelve weeks, the leaves above and below the inoculation points of plants inoculated with mutant strains that were showing disease symptoms were collected and tested for the presence of the bacterium using nested PCR as described by Botha et al. (2001). Alongside these, the leaves collected from the plants inoculated with corresponding complemented strains were also tested for the presence of the bacterium. Briefly, the plant material was crushed in sterile distilled water, incubated at room temperature for approximately one hour followed by two washes with a wash buffer (100 mM Tris-HCl; 20 mM $NaCl_2$, pH 8.8), final resuspension and boiling in the same buffer containing minuscule traces of insoluble polyvinylpolypyrrolidone (PVPP) (EMD Chemicals Inc. New Jersey, USA). In the first PCR, the 16S-23S rDNA spacer region was amplified using the universal primers A1 and B1 (Barry et al. 1991). A 277 bp fragment within the 16S-23S rDNA spacer region was subsequently amplified using 1 µl of the initial PCR reaction as a template with primers S3 and S4 (Botha et al. 2001). Following PCR, gel electrophoresis analysis was performed on a 0.8% agarose gel.

Infected shoots were harvested after weeks four, eight and twelve. To observe the role of T4P in migration of bacteria and colonization of the tissues away from the inoculation point, 1 cm longitudinal pieces were cut out of the shoot at the inoculation point as well as at 5 cm below and above the inoculation point. The prepared grapevine shoot pieces were fixed using a fixative (4 % glutaraldehyde and 2.5 % paraformaldehyde prepared in a phosphate buffer pH 7.4 at a 1:1 ratio) and incubated overnight at 4 °C. After incubation, samples were washed twice with phosphate buffer pH 7.4 and chilled at 4 °C. To dry the samples, 20-minute washes with ethanol solutions of increasing concentration (30%, 50%, 60%, 70%, 90%, 96%, and 100%) were performed at room temperature. The samples were critically dried in a ventilated oven set at 22 °C for three days. The prepared samples were analysed for the presence of bacteria using a scanning electron microscope (SEM). To prepare the sample for SEM viewing, the samples were mounted on SEM sample stubs and coated with a thin layer of carbon nanoparticles. The InLens detector micrographs were acquired using the Zeiss MERLIN Field Emission Scanning Electron Microscope at the Electron Microbeam Unit of Stellenbosch University's Central Analytical Facility. Beam conditions during

imaging were 2 kV acceleration voltage, 250 pA probe current, with a working distance of approximately 4mm.

3.3. RESULTS

3.3.1. T4P Gene Sequence Analysis

The *X. ampelinus* VS20 T4P open reading frames were identified using the *A. avenae* T4P protein sequences. Protein sequence comparison revealed almost 100% sequence similarity between *X. ampelinus* VS20 and *X. ampelinus* CECT 7646, while *Xylophilus* sp. Leaf220, isolated during a study on Arabidopsis, shared 96-99% similarity for all genes except *pilA*. The T4P proteins of *Acidovorax*, *Ralstonia*, *Xanthomonas*, *Pseudomonas*, and *Xylella* species shared a much lower level of sequence similarity with *X. ampelinus* with the highest similarity occurring in the conserved regions of the proteins.

A conserved protein domain search for *X. ampelinus* T4P proteins using the SmartEMBL online sequence analysis tool revealed that these sequences have the conserved domains typical of T4P and T2S (type II secretion) systems (Figure 3.1). The type IV pilin protein, PilA has a signalling peptide and a pilin domain found in all pilin subunits that are precursors of the pilus filament. PilB, the assembly ATPase contains a T2SSE_N and the AAA domains found at the N-terminus of members of the type II secretion system protein E and in ATPases associated with a variety of cellular activities including type IV pilus assembly. Inner membrane T2SS F family platform protein, PilC has the T2SSF domain representing a group of proteins that form the platform for the machinery of the T2SS system as well as T4P and archaeal flagella.

The Type IV prepilin-like proteins leader peptidase-processing enzyme, PilD, has a peptidase domain found in protein families involved in post-translational modification of proteins such as methylation of pilins prior to their secretion as active proteins. T4P outer membrane protein, PilQ, carries a secretin domain, which functions in diverse protein export and secretion pathways. In addition to this domain, PilQ also has a signal peptide and TonB N terminus (STN) short domain found at the N-terminus of the secretins of the bacterial type II/III secretory system as well as the TonB-dependent receptor proteins involved in TonB-dependent active uptake of selected substrates. Finally, twitching motility protein and retraction ATPase PilU has a T2SSE domain,

which is also found in proteins involved in the Type II protein secretion system (T2SS) and Type IV protein secretion system (T4SS) of Gram-negative bacteria.

Comparative analysis of the genetic organization of T4P genes shows that the three *Xylophilus* isolates have the same arrangement of genes as *A. avenae* subsp. *avenae* (Figure 3.2). The *pilBCD* gene cluster of *Xylophilus* is also found in *A. avenae* subsp. *avenae* and *Ralstonia solanacearum*. In both *X. citri* pv. *citri* and *X. fastidiosa*, the *pilB* gene is found together with response regulators *pilR* and *pilS*. A striking difference in the organization of the disassembly and retraction ATPases PilU and PilT of *X. ampelinus* VS20, *X. ampelinus* CECT 7646, *Xylophilus* sp. Leaf220, and *A. avenae* is that *pilU* and *pilT* genes are separated by a cyclic AMP (cAMP) receptor protein (CRP) belonging to the *crp/fnr* family of transcriptional regulators, which is absent in the other pathogens investigated. Another difference of note is the location of *pilE* downstream of *pilU* in *P. syringae* pv. *tomato*. While other genes investigated in this study were within the operon-like clusters associated with T4P, the *pilA* gene was located downstream of the gene encoding the adenylate/guanylate cyclase domain-containing protein with general function in intracellular signal transduction.

3.3.2. Validation of Mutant and Complementation Constructs

T4P gene-deletion mutants were confirmed by the absence of each gene and the insertion of a kanamycin-resistance cassette at each T4P gene locus (Figure 3.3). PCR amplification of the kanamycin gene and the promoter region of each T4P gene was done to confirm gene replacement. The complemented strains were validated through PCR amplification of the gene and its promoter within the multiple cloning site of the broad host range vector pBBR1MCS5 (Figure 3.4).

3.3.3. Scanning Transmission Electron Microscopy (STEM)

Phenotypic confirmation of the effect of T4P gene deletion was obtained through STEM analysis of the mutants (Figure 3.5). As expected, wild-type *X. ampelinus* expressed multiple pilus fibres extending out from the cell envelope (Figure 3.5). T4P were completely absent in all mutant strains except for the *pilU* mutant strain which was slightly piliated, which was not unexpected due to the absence of ATPase activity required for pilus disassembly (Figure 3.5). Complementation of the

mutant strains with corresponding T4P genes and their native promoters in pBBR1MCS5 restored wild-type phenotype (Figure 3.5).

3.3.4. *In vitro* Characterization of T4P Genes

3.3.4.1. *Twitching Motility*

All mutant and complemented strains were tested for their twitching motility phenotype. As expected, the ability of the cells to move via twitching - displayed by the fringe on the colony margin with striations - was not observed for the T4P mutants; instead, a smooth sharp colony edge without twitching zones was observed (Figure 3.6). Complementation of the mutant strains resulted in twitching zones comparable to the wild-type cells. There was, however, no significant change in colony sizes across strains.

3.3.4.2. *Biofilm Formation*

Microscopy analysis of the biofilm structures formed, shows that the *pilA*, *pilB*, *pilC*, and *pilD* T4P mutant strains formed reduced biofilms on glass slide coverslips, while the *pilQ* and *pilU* mutant strains formed visually intense biofilms on glass surfaces comparable to the wild-type during both the initial attachment and mature biofilm stages (Figure 3.7 and 3.8). A definite micro-colony and biofilm structure were observed for the wild-type and complemented strains compared to the dispersed biofilm structure shown by mutant strains. Complementation of the T4P deletion mutants restored the wild-type phenotype.

3.3.5. The Role of T4P in Virulence

Three weeks post-inoculation, callus formation was observed at the point of inoculation for all infected plants. Swelling and necrosis around the wound was observed for only the plants infected with the wild-type and complemented strains. Symptom manifestation was observed on leaves from the fourth-week post-inoculation. The leaves above the point of inoculation of the plants infected with all complemented strains except *XapilQc* developed reddish-brown discolouration, characteristic of bacterial blight disease symptoms on leaves, followed by drying up of such leaves while the ones below the inoculation point remained healthy (Figure 3.9). No symptoms were observed on the plants inoculated with mutant strains except for *XaΔpilD* and *XaΔpilU* mutant

strains, which showed brown-discoloration coupled with the drying of such leaves. At the sixth-week post inoculation, plants inoculated with the wild-type, *XapilAc*, *XapilBc*, *XapilCc*, *XapilDc*, and *XapilUc*, that were initially showing leaf symptoms, developed cankers that extended from the point of inoculation along the shoot length (Figure 3.10). Some formed cracks at the base of the leaf petioles above the inoculation point. At this point, the reddish-brown discoloration of leaves was also observed on plants inoculated with the wild-type isolate (Figure 3.9). At the end of twelve weeks, plants inoculated with complemented strains (*XapilAc*, *XapilBc*, *XapilCc*, *XapilDc*, and *XapilUc*), and the wild-type showed differing levels of symptom severity with respect to discolouration of leaves and canker length (Figure 3.10), while plants inoculated with the *XaΔpilD* and *XaΔpilU* mutants only showed symptoms on leaves but did not form cankers. However, the leaf petioles of the plants inoculated with *XaΔpilU* also showed cracks at the base in addition to necrosis on the leaf blades (Figure 3.10). None of the plants showed symptoms on leaves below the point of inoculation at twelve weeks post inoculation. The *XapilQc* complemented strain failed to produce symptoms on plants.

To determine whether the leaf symptoms were due to the migration of the inoculated bacterial strains, nested PCR of symptomatic leaves above and asymptomatic leaves below (from only the symptomatic plants) the inoculation point was conducted to confirm the presence of *X. ampelinus* (Table 3.5). Results showed that at twelve weeks post-inoculation, leaves from plants inoculated with the wild-type, the *XaΔpilU* mutant and complementation strains *XapilAc*, *XapilBc*, *XapilCc*, *XapilDc*, and *XapilUc* tested positive for *X. ampelinus* in leaves above and below the point of inoculation. Only the symptomatic leaves above the point of inoculation from plants inoculated with the *XaΔpilD* mutant tested positive for the presence of *X. ampelinus.*

3.3.6. *In planta* Biofilm Formation and Host Colonization

Biofilm development and tissue colonization were evaluated to investigate the role of T4P in plant-pathogen interaction using scanning electron microscopy. At four weeks post-inoculation, cells were only observed at the inoculation point for all strains and not at a five-centimetre distance away from the inoculation point. At this time point, five of the T4P mutant strains failed to form biofilms at the point of inoculation. A few isolated cells were observed in the xylem vessels of the infected shoots, while dense biofilms were observed for the wild-type and complemented strains

(Figure 3.11). As expected (based on the *in vitro* results), the *XaΔpilU* mutant strain formed biofilms comparable to the wild-type (Figure 3.11).

At eight weeks-post inoculation, microcolony formation was impaired for all mutants except for *XaΔpilU,* which formed dense biofilms comparable to those formed by the wild-type cells (Figure 3.12). None of the mutant strains were observed at a five-centimetre distance away from the inoculation point. As expected, the wild-type, *XaΔpilU* mutant, and complemented strains formed biofilms at the point of inoculation and a few cells were also observed at the five-centimetre distance away from the inoculation point, even though no biofilms were observed at this point.

Twelve weeks post-inoculation, the examination of a longitudinal section of the infected shoots at the inoculation point confirmed the reduction of cell aggregates in plant xylem vessels inoculated with all strains except *XapilQc* (Figure 3.13). It was also noticeable that while all other inoculated plant tissues harvested at the point of inoculation, showed drying and reduced numbers of bacterial cells, tissue from plants inoculated with *XapilQc* remained green and healthy even after 12 weeks (Figure 3.10). At this time point, biofilm formation was observed at a distance of five centimetres away from the inoculation point, in the xylem vessels of plants inoculated with wild-type *X. ampelinus* and all complemented strains except for *XapilDc* and *XapilQc* (Figures 3.14 and 3.15). Five mutant strains deficient in T4P failed to colonize and form biofilms at a five-centimetre distance below and above the inoculation point, 12 weeks post-inoculation. The *XaΔpilU* strain cells expressing T4P were discovered at a five-centimetre distance above and below the point of inoculation but no signicant biofilm was formed by this strain when compared to those formed by its complemented strain *XapilUc* as well as the wild-type (Figures 3.14 and 3.15). When assessing biofilm structures formed by these cells, the wild-type, *XaΔpilU* mutant and five of the complemented strains (*XapilAc*, *XapilBc*, *XapilCc*, *XapilQc*, and *XapilUc*), with the exception of *XapilDc* produced noticeable amounts of extracellular components of the biofilm matrix surrounding the biofilm cells (Figures 3.11 and 3.12).

3.4. DISCUSSION

Xylophilus ampelinus colonizes grapevine plants and forms biofilm aggregates (Grall and Manceau 2003), however, the mechanism by which this pathogen colonizes the interior of infected plants and forms biofilms in the xylem vessels and how biofilms influence virulence is not yet understood. The previous model of *Xylella fastidiosa* showed that T4P-mediated surface motility is required for upstream migration of the bacteria (Meng et al. 2005). Here in this study, the role of T4P in surface colonization as well as *in planta* colonization was investigated. Due to the limited availability of genetic information for the pathogen, the T4P complex genes were initially identified using the sequences from the *Acidovorax avenae* subsp. *avenae* and were validated by comparing the sequences with those of other closely related phytopathogens reported to express T4P.

Six T4P genes (*pilA*, *pilB*, *pilC*, *pilD*, *pilQ*, and *pilU*) with roles in T4P assembly and function (as reported for other pathogens) were selected for study in *X. ampelinus.* Sequence analysis of the retraction ATPases in *X. ampelinus* revealed that there are at least two copies of *pilT* and one *pilU.* The two copies of the PilT found in each of the *X. ampelinus* strains (VS20 and CECT 7646) share 36% similarity, suggesting that these proteins may differ in function, however, further functional characterization and confirmation is required.

As reported in other species of bacteria such as *P. aeruginosa* and *A. citrulli*, the *X. ampelinus pilU* gene is located downstream of *pilT* (Rosenberg et al. 2018; Whitchurch and Mattick 1994). Both *pilT* and *pilU* control the twitching motility function of T4P via pilus retraction (Mattick 2002). PilU differs from PilT in the presence of type II secretory pathway ATPase GspE/PulE or T4P pilus assembly pathway ATPase PilB domain, which functions in cell motility as well as in intracellular trafficking, secretion, and vesicular transport. This conserved domain in PilU is very different from the pilus retraction ATPase domain (PilT P-loop NTPase) in PilT, which is involved in cell motility and in other diverse cellular functions, suggesting that in addition to twitching motility, the two proteins could also play other roles in cellular functions.

Following mutation and complementation of these genes, phenotypic validation was done through STEM analysis to observe the role of each gene in T4P expression. As found in other bacterial pathogens, five *X. ampelinus* T4P gene deletion mutant strains (*XaΔpilA*, *XaΔpilB*, *XaΔpilC*, *XaΔpilD*, and *XaΔpilQ*) were deficient in T4P formation. This phenotype is commonly observed

in some of the T4P gene mutants in other plant pathogens such as *A. avenae*, *X. fastidiosa*, and *Ralstonia solanacearum*, and *Pantoea ananatis* (Bahar et al. 2009; Kang et al. 2002; Meng et al. 2005; Weller-Stuart et al. 2017). Failure of these mutants to express extracellular T4P fibres indicates that *pilA*, *pilB*, *pilC*, *pilD*, and *pilQ* are indispensable for pilus assembly in *X. ampelinus*. The *pilU* mutant expressed pili in a manner similar to the wild-type strain. Based on the STEM images (Figure 3.5), the cells were not hyper-piliated as have been reported for *pilU* mutants of *P. aeruginosa* (Whitchurch and Mattick 1994), but were however piliated, a phenotype also observed for the human pathogen, *Neisseria gonorrhoeae, pilU* mutant (Park et al. 2002), as opposed to the non-piliated *pilU* mutant of plant pathogen, *A. citrulli* (Rosenberg et al. 2018). Failure to express (*XaΔpilA*, *XaΔpilB*, *XaΔpilC*, *XaΔpilD*, and *XaΔpilQ*) and failure to retract (*XaΔpilU*) functional T4P resulted in a phenotype with reduced surface twitching for all mutant strains on a soft-agar surface producing only the sharp, smooth-edged colonies without twitching zones. Because of the slow-growth characteristic of the bacterium, the twitching zones observed at day ten post-inoculation were less defined. However, smoother colony morphology was observed for all mutants compared to the rough colony surface with rough edges of the wild-type and complemented strains confirming the importance of T4P in cell twitching motility (Mattick 2002; Merz et al. 2000). The twitch-minus phenotype of the piliated *pilU* mutant confirms the role of *pilU* in pilus retraction and that pilus retraction function of the T4P drives twitching motility.

Functionally, T4P are implicated in attachment and micro-colony formation in many bacterial species including *X. citri* subsp. *citri* and *Neisseria gonorrhoeae* (Dunger et al. 2014; Merz et al. 2001; Park et al. 2002). To test for this fact in *X. ampelinus*, an *in vitro* biofilm assay was conducted to assess the function of T4P during the initial and mature biofilm stages of biofilm formation. As reported for other pathogens, mutations in *pilA*, *pilB*, *pilC*, and *pilD* resulted in the impaired ability of the bacterial cells to form biofilms *in vitro*, indicating that *X. ampelinus* require T4P to form micro-colonies and aggregate to form a bigger biofilm structure. In the few reported instances, *pilU* has been shown to have varying effects on biofilm formation. The *Acidovorax citrulli pilU* mutant expresses a non-piliated phenotype and can form neither biofilms nor cause disease (Rosenberg et al. 2018), whereas the piliated *N. gonorrhoeae pilU* mutants retained adherence to the human epidermoid carcinoma cells (Park et al. 2002). Similar to the latter observation, the *X. ampelinus* piliated *pilU* mutant formed biofilms and retained the wild-type phenotype *in vitro*.

Because T4P are adhesive in nature, the presence of the T4P on the surface of this mutant (although not functional in terms of twitching motility) may have contributed to the formation of wild-type-like biofilms by the *pilU* mutant. Unexpectedly, the non-piliated T4P *pilQ* mutant also formed dense biofilms at the point of inoculation that are comparable to those formed by the wild-type cells. Similar results were observed for the *X. oryzae* pv. *oryzae pilQ* mutant that attached to *in vitro* glass surfaces in a manner similar to the wild-type cells (Das et al. 2009). This *pilQ* mutant phenotype suggests that in some species, mutations in the *pilQ* gene may not have any significant effect in the ability of the bacterial cells to form biofilm *in vitro*.

Because the purpose of this study was to examine the biofilm formation process of *X. ampelinus* in plants, the role of T4P in the formation of *in planta* biofilms and colonization was investigated using SEM, nPCR, and by visual observation of symptom development. Fewer mutant cells observed at the points of inoculation at four, eight and twelve weeks indicate that the establishment of *X. ampelinus* inside the plants is dependent upon the adhesiveness of the T4P to attach to surfaces and twitching motility function of the T4P to develop biofilms. As expected, the T4P-expressing *pilU* mutant formed biofilms comparable to those formed by the wild-type at the point of inoculation, indicating that when expressed, the T4P could still promote attachment to the surface due to its natural adhesiveness. Through SEM analysis of the plant vasculature, it was shown that *X. ampelinus* requires T4P to migrate inside the plants as *pilA*, *pilB*, *pilC*, and *pilQ* mutants deficient in T4P-expression were unable to move from the point of inoculation to distal parts of the plants. Although a few *pilD* and *pilU* mutant cells were detected at a 5 cm distance away from the inoculation point, the inoculated grapevines exhibited milder symptoms compared to those inoculated with the wild-type. This indicates that the ability of the cells to translocate within the host is associated with virulence as was observed for the wild-type and complemented strains with full ability to move. Although the *pilQ* complement was able to form biofilms at the point of inoculation, the unexpected failure of this mutant to translocate from the point of inoculation resulted in an avirulent phenotype also indicating that the virulence of *X. ampelinus* is dependent on the ability of the bacteria to translocate inside the plants. The failure of the pilD complement to form biofilms at the 5 cm distance away from the inoculation at week 12 was unexpected since at week 8 few *XapilDc* strain cells were present at this site (result not shown). The presence of these cells was also confirmed by the positive nPCR result and the virulent

phenotype exhibited by the *XapilDc* strain, indicating that the bacteria were able to translocate within the plants regardless of the negative SEM biofilm result at 5 cm distance. Furthermore, the full virulence exhibited by the wild-type and complemented strains that formed biofilms at distal parts from the inoculation point, indicates that T4P-dependent twitching motility facilitates biofilm formation in plants, which thereby, contributes to full virulence. This concept was further confirmed by the failure of the *pilU* mutant cells to form micro-colonies and biofilms at a 5 cm distance away from the inoculation point even though the cells were present at this point (as detected by nPCR and SEM). Consequently, the observed role of T4P in biofilm development inside plants and the reduced virulence exhibited by all the T4P mutants indicate that T4P are required for *in planta* biofilm formation and surface colonization through twitching motility and ultimately T4P are required for the virulence of *X. ampelinus* in its host, grapevine.

Reduction in the virulence of T4P mutants has been observed in other plant pathogenic bacteria, including *Xylella fastidiosa*, *Pseudomonas syringae* pv. *tabaci*, *Ralstonia solanacearum*, *Xanthomonas oryzae pv. oryzae*, and *Acidovorax* species (Bahar et al. 2009; Das et al. 2009; Kang et al. 2002; Meng et al. 2005; Taguchi and Ichinose 2011; Rosenberg et al. 2018). Reduction of virulence of T4P mutants suggests that T4P play a role in disease development in plants. Previous studies also show that the expression and function of T4P greatly correlate with the ability of the pathogens to colonize their plant hosts (Kang et al. 2002; Meng et al. 2005; Siri et al. 2014; Taguchi and Ichinose 2011). Wang et al. (2008), through transposon mutagenesis, also showed that the *pilQ* gene, among others, play an important role in the virulence in *X. oryze* pv. *oryzae*. This finding also supports our observation despite the fact that the *X. ampelinus pilQ* complemented strain only formed biofilms at the point of inoculation; its mutant was, however, avirulent and failed to form biofilms in plants, irrespective of its ability to attach and form biofilms on glass surfaces, indicating that *pilQ* is required for virulence in *X. ampelinus*. According to Das et al. (2009), the *X. oryze* pv. *oryzae pilQ* mutant showed reduced virulence despite its ability to attach to glass surfaces. Similar findings were also reported for *R. solanacearum* and *X. fastidiosa pilQ* mutants (Liu et al. 2001; Meng et al. 2005). Meng et al. (2005) also reported an avirulent phenotype expressed by *pilB* mutants, similar to that observed in this study. As observed in our study, inactivation of a major pilin subunit *pilA* gene in *R. solanacearum* reduced the virulence of the *pilA* mutant strain (Kang et al. 2002). Furthermore, the *pilA* mutations in two *Pseudomonas* species *P. syringae* pv. *tabacci*

and *P. syringae* pv. *syringae* proved the role of *pilA* in virulence and field fitness, respectively (Roine et al. 1998; Taguchi and Ichinose 2011). Previously, Kang et al. (2002) proved that strains carrying *pilA* mutations fail to express the PilA protein extracellularly indicating that in the absence of PilA pilin subunits the T4P are not formed, which affects attachment, biofilm formation, and subsequently virulence. Recently, a screen of the transposon mutants of *A. citrulli* also revealed among T4P genes, *pilB*, *pilQ*, and *pilU* mutants with impairment in disease development (Rosenberg et al. 2018). The phenotypes with reduced virulence observed for T4P-deficient *pilA*, *pilB*, *pilC*, *pilD*, and *pilQ* mutants investigated in this study suggest that the T4P are required for the virulence in *X. ampelinus*. Although all the T4P-deficient mutant strains were reduced in virulence, the appearance of disease symptoms - albeit less severe than the wild-type - observed in plants inoculated with *XaΔpilD* cannot be ignored. *P. syringae* pv. *tomato* DC3000 tomato interaction, it was shown that *pilD* mutants had little effect on leaf interactions, implying that deletion of *pilD* did not affect pathogenicity in this system (Roine et al. 1998). However, the *XaΔpilD* phenotypic display in reduced twitching motility and biofilm formation arising from T4P-deficiency still confirms the role of *pilD* role in pilus assembly in *X. ampelinus*.

3.5. CONCLUSION

Biofilm formation is one of the major pathogenicity factors employed by phytopathogenic bacteria to cause disease. However, the process of biofilm formation in grapevine by *X. ampelinus* has remained unknown. Here we report the role of T4P-mediated twitching motility in host colonization, *in planta* biofilm formation, and virulence of *X. ampelinus*. Characterization of the selected six genes revealed the importance of T4P in the establishment of the pathogen in grapevine, biofilm formation and virulence. This is the first report on functional characterization of factors contributing to biofilms formed by *X. ampelinus*, which also addresses the role of T4P during biofilm formation in plants.

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TABLES

Table 3.1. Bacterial strains and plasmids used in this study

Bacterial strains and plasmids	Relevant characteristics	Reference/ Source
Bacterial Strains		
Escherichia coli JM109	*endA1, recA1, gyrA96, thi, hsdR17 (rk–, mk+), relA1, supE44, Δ(lac-proAB), [F´ traD36, proAB, laqIqZΔM15].*	Lab strain
Xylophilus ampelinus VS20	Wild-type *Xylophilus ampelinus*	ARC-PPRI[a] Culture collection
XaΔpilA	*X. ampelinus pilA* gene knockout mutant; Kan^r	This study
XaΔpilB	*X. ampelinus pilB* gene knockout mutant; Kan^r	This study
XaΔpilC	*X. ampelinus pilC* gene knockout mutant; Kan^r	This study
XaΔpilD	*X. ampelinus pilD* gene knockout mutant; Kan^r	This study
XaΔpilQ	*X. ampelinus pilQ* gene knockout mutant; Kan^r	This study
XaΔpilU	*X. ampelinus pilU* gene knockout mutant; Kan^r	This study
XapilAc	*X. ampelinus pilA* complementation construct; Kan^r; Gm^r	This study
XapilBc	*X. ampelinus pilB* complementation construct; Kan^r; Gm^r	This study
XapilCc	*X. ampelinus pilC* complementation construct; Kan^r; Gm^r	This study
XapilDc	*X. ampelinus pilD* complementation construct; Kan^r; Gm^r	This study
XapilQc	*X. ampelinus pilQ* complementation construct; Kan^r; Gm^r	This study
XapilUc	*X. ampelinus pilU* complementation construct; Kan^r; Gm^r	This study

Plasmids

pJET1.2/blunt	Rep (pMB1) replicon, eco47IR lethal gene, PlacUV5 modified Plac promoter, T7 RNA polymerase promoter; Amp^r	ThermoFisher (Waltham, Massachusetts, USA)
pJETΔ*pilA*	pJET1.2/blunt carrying *pilA* SOEing product (sequences downstream and upstream of the *pilA* open reading frame flanking the kanamycin gene) Amp^r; Kan^r	This study
pJETΔ*pilB*	pJET1.2/blunt carrying *pilB* SOEing product (sequences downstream and upstream of the *pilB* open reading frame flanking the kanamycin gene) Amp^r; Kan^r	This study
pJETΔ*pilC*	pJET1.2/blunt carrying *pilC* SOEing product (sequences downstream and upstream of the *pilC* open reading frame flanking the kanamycin gene) Amp^r; Kan^r	This study
pJETΔ*pilD*	pJET1.2/blunt carrying *pilD* SOEing product (sequences downstream and upstream of the *pilD* open reading frame flanking the kanamycin gene) Amp^r; Kan^r	This study
pJETΔ*pilQ*	pJET1.2/blunt carrying *pilQ* SOEing product (sequences downstream and upstream of the *pilQ* open reading frame flanking the kanamycin gene) Amp^r; Kan^r	This study
pJETΔ*pilU*	pJET1.2/blunt carrying *pilU* SOEing product (sequences downstream and upstream of the *pilU* open reading frame flanking the kanamycin gene) Amp^r; Kan^r	This study
pBBR1MCS5	Broad-host-range vector, MCS, *LacZ* alpha peptide, *RK2*, *IncP*, *IncQ*, *IncW*, *ColE1* and *P15a*-based replicons; Gm^r	Kovach *et al*., 1995
pBMCS5*pilAc*	pBBR1MCS5 vector carrying full-length *pilA* coding region and its native promoter Gm^r	This study
pBMCS5*pilBc*	pBBR1MCS5 vector carrying full-length *pilB* coding region and its native promoter Gm^r	This study
pBMCS5*pilCc*	pBBR1MCS5 vector carrying full-length *pilC* coding region and its native promoter Gm^r	This study
pBMCS5*pilDc*	pBBR1MCS5 vector carrying full-length *pilD* coding region and its native promoter Gm^r	This study
pBMCS5*pilQc*	pBBR1MCS5 vector carrying full-length *pilQ* coding region and its native promoter Gm^r	This study
pBMCS5*pilUc*	pBBR1MCS5 vector carrying full-length *pilU* coding region and its native promoter Gm^r	This study

[a] ARC PPRI: Agricultural Research Council, Plant Protection Research Institute

Amp^r: Ampicillin resistant; Kan^r: Kanamycin resistant; Gm^r: Gentamicin resistant

Table 3.2. Primers used for SOEing, complementation, validation and sequencing

Primer name	Primer sequence* 5′-3′	Product size	Annealing temperature
	SOEing PCR primers		
PilAUF	AATCGGCATCGGGATCGGCAT		
PilAUR	ACAATTCATCGATGATGGTTGTTATCAGCAGCTTCCATGCCA	720 bp	58 °C
PilAKanF	TGGCATGGAAGCTGCTGATAACAACCATCATCGATGAATTGT		
PilAKanR	ATATCGGAAATCAAGTGCTATTAGAAAAACTCATC	926 bp	58 °C
PilADF	GATGAGTTTTTCTAATAGCACTTGATTTCCGATAT		
PilADR	ACGGATTACAACTTCAAGCGGA	809 bp	58 °C
PilBDF	GAACTCGCTCATCGCGATCACGATCA		
PilBDR	AGAATTGGTTAATTGGTTCGTACGACAACCCGCGAT	700 bp	65 °C
PilBKanF	ATCGCGGGTTGTCGTACGAACCAATTAACCAATTCT		
PilBKanR	TCTTCCGGTCCCCACGGCCAACCATCATCGATGAATTGT	926 bp	65 °C
PilBUF	ACAATTCATCGATGATGGTTGGCCGTGGGGACCGGAAGA		
PilBUR	ACCTTGCCGGCCTTGGCGGCCAAGT	700 bp	60 °C
PilCDF	AGCGCCTGCCAGCCGAACCA		
PilCDR	AGAATTGGTTAATTGGTTTGTCGCCGGAGATCGACAT	693 bp	60 °C
PilCKanF	ATGTCGATCTCCGGCGACAAACCAATTAACCAATTCT		
PilCKanR	TCATCGGAGAAAGTCATCAACCATCATCGATGAATTGT	926 bp	60 °C
PilCUF	ACAATTCATCGATGATGGTTGATGACTTTCTCCGATGA		
PilCUR	AGACGGTGTCGCTCTACACCT	729 bp	58 °C
PilDDF	TCCATGATCTCGAAGATCGAGGCCA		
PilDDR	AGAATTGGTTAATTGGTTAGACGCCACAGGGCCGCTGGCA	774 bp	60 °C
PilDKanF	TGCCAGCGGCCCTGTGGCGTCTAACCAATTAACCAATTCT		
PilDkanR	AAGCTCGGCCAAGTCGTCTCAACCATCATCGATGAATTGT	926 bp	60 °C
PilDUF	ACAATTCATCGATGATGGTTGAGACGACTTGGCCGAGCTT		
PilDUR	AGGCGATCAAGTCGAAGATCCGCT	734 bp	60 °C
PilQUF	ACGCTCAACAACCTCTCCATTTC		
PilQUR	ACAATTCATCGATGATGGTTGCGCCGGGACCAGTTTTTCAT	701 bp	60 °C
PilQKanF	ATGAAAAACTGGTCCCGGCGCAACCATCATCGATGAATTGT		
PilQKanR	GCGAATGCAAGAAATCCCCGTTAGAAAAACTCATC	926 bp	60 °C
PilQDF	GATGAGTTTTTCTAACGGGGATTTCTTGCATTCGC		
PilQDR	GCCAAGCGACTGATAAACCG	622 bp	58 °C

PilUUF	CACGCCCCCACCGCACGCGCATTCGA		
PilUUR	ACAATTCATCGATGATGGTTGTCGGTCGTCCTCGGAGGG	700 bp	65 °C
PilUKanF	CCCTCCGAGGACGACCGACAACCATCATCGATGAATTGT		
PilUKanR	AAGGCGTAGAAAGCCCCTTGTTAGAAAAACTCATC	926 bp	60 °C
PilUDF	GATGAGTTTTTCTAACACAAGGGGCTTTCTACGCCTT		
PilUDR	GTGGTGTGGTCGACGAAGAT	555 bp	58 °C

T4P complementation primers

PilAF	ATATCTAGATGTCAACTCGTATCTTTGT		
PilAR	TCAGGATCCTTATGTAGCAGTACTGGTGCA	589 bp	58 °C
PilBF	TATGAGCTCAGGAGGCTCCTGAGACCGCA		
PilBR	TTATCTAGATTATTCGTTGGTGACCGCGAGCA	1978 bp	65 °C
PilCF	ATATCTAGATCGAAGGCGTGCGCTCCCTG		
PilCR	TCAGGATCCTCAGACGACTTGGCCGAGCTT	1352 bp	60 °C
PilDF	ATATCTAGAGCGAAGTCGACGAGATGGT		
PilDR	TCAGGATCCTCAGAAGCCCAGCAGCCGCAAT	1008 bp	65 °C
PilQF	ATATCTAGAGCCAACGAAACGCTGATCAC		
PilQR	TCAGGATCCTCACCGGGCAGCGGTGCGGT	2450 bp	65 °C
PilUF	ATATCTAGAGTCGCTGCTCGACAGCGGCA		
PilUR	TCAGGATCCTCAGACGATGGCGAAATGCT	1369 bp	60 °C

Vector validation primers (flanking pBBR1MCS5 LacZ operon)

YP320	TTACAATTTCCATTCGCCATTCAG		
YP321	ATGACCATGATTACGCCAAGC	366 bp	53 °C

***Xylophilus ampelinus* 16S-23S rDNA spacer region Nested PCR primers**
(Barry et al. 1991; Botha et al. 2001)

A1	AGTCGTAACAAGGTAAGCCG		
B1	CYRYTGCCAAGCATCCACT	742 bp	52 °C
S3	GGTGTTAGGCCGAGTAGTGAG		
S4	GGTCTTTCACCTGACGCGTTA	277 bp	52 °C

Sequencing primers (CloneJET PCR Cloning Kit, This study)

pJET1.2F	CGACTCACTATAGGGAGAGCGGC		
pJET1.2R	AAGAACATCGATTTTCCATGGCAG	N/A	N/A
KanF	CAACCATCATCGATGAATTG		

KanR	TTAGAAAAACTCATCGAG	N/A	N/A

* Restriction enzyme sites are underlined

* N/A - The amplicon sizes produced by pJET1.2 sequencing primers were dependent on the size of the cloned insert.

Table 3.3. Generic SOEing PCR

Component	Master Mix A	Master Mix B
5X Phusion buffer	5 µl	5 µl
10 mM dNTPs	0.5 µl	0.5 µl
Phusion polymerase	0.25 µl	0.25 µl
10 µM external forward primer	0 µl	1 µl
10 µM external reverse primer	0 µl	1 µl
DNA template A	20 ng/µl	0 µl
Kanamycin cassette	20 ng/µl	0 µl
DNA template B	20 ng/µl	0 µl
Nuclease-free water	up to 25 µl	up to 25 µl
TOTAL	**25 µl**	**25 µl**

*DNA template A= Sequence upstream of T4P gene CDs

*DNA template B= Sequence downstream of T4P gene CDs

Table 3.4. BLASTp pairwise disparity of *X. ampelinus* VS20 T4P protein sequences to other plant pathogens

T4P Protein	*X. ampelinus* CECT 7646	*Xylophilus* sp. Leaf220	*A. avenae* subsp. *avenae*	*R. solanacearum*	*P. syringae* pv. *tomato*	*X. fastidiosa Temecula1*	*X. citri pv. citri*
PilA	**99%** WP_110466449.1	**40%** WP_082448667.1	**57%** AVS89343.1	**35%** WP_080981129.1	**47%** WP_011103296.1	**37%** WP_004090375.1	**56%** WP_080954329.1
PilB	**100%** PYE78331.1	**96%** KQM71227.1	**80%** AVS63106.1	**60%** WP_014616069.1	**50%** WP_011103350.1	**52%** WP_004090374.1	**51%** WP_041471193.1
PilC	**100%** PYE78330.1	**97%** KQM71228.1	**86%** AVS63107.1	**60%** WP_013211405.1	**51%** WP_011103349.1	**48%** WP_080502631.1	**48%** WP_040268844.1
PilD	**100%** PYE78329.1	**96%** KQM71229.1	**96%** AVS89449.1	**53%** WP_055324789.1	**53%** WP_005770019.1	**45%** WP_007962469.1	**42%** WP_004084594.1
PilQ	**100%** PYE78254.1	**99%** KQM71294.1	**71%** AVS69659.1	**50%** WP_020747317.1	**40%** WP_007245595.1	**37%** WP_011098213.1	**37%** WP_045715706.1
PilU	**100%** PYE75848.1	**99%** KQM70246.1	**89%** ADX44528.1	**69%** WP_021195931.1	**58%** WP_011104340.1	**57%** WP_011097971.1	**62%** WP_057675908.1

Table 3.5. *Xylophilus ampelinus*-specific nested PCR of leaf material from grapevines inoculated with type IV pili mutants and their complemented strains

Strain	Above the IP[a]	Below the IP
wt *X. ampelinus*	+	+
XaΔpilD	+	-
XaΔpilU	+	+
XapilAc	+	+
XapilBc	+	+
XapilCc	+	+
XapilDc	+	+
XapilUc	+	+

[a] IP: inoculation point;

+ tested positive for *X. ampelinus*

- tested negative for *X. ampelinus*

FIGURES

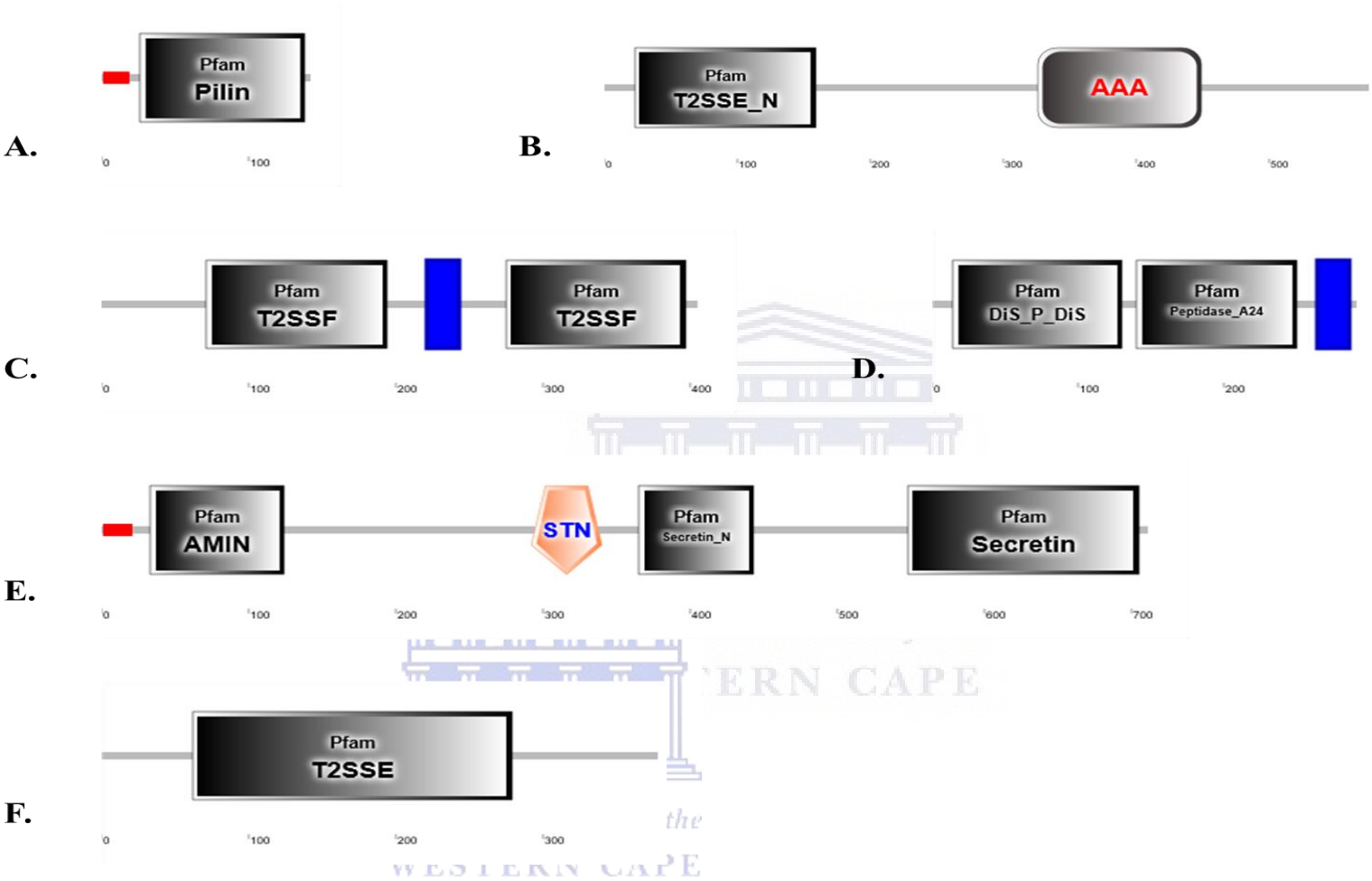

Figure 3.1. Conserved domains of *X. ampelinus* VS20 T4P proteins generated using the SMART EMBL online tool (http://smart.embl-heidelberg.de/). The conserved domains are displayed in blocks. The blue and small red blocks indicate the transmembrane regions and signalling peptides, respectively. The numbers below the conserved protein domains indicate the amino acid numbers. A.) PilA, B.) PilB, C.) PilC, D.) PilD, E.) PilQ, and F.) PilU protein family domains.

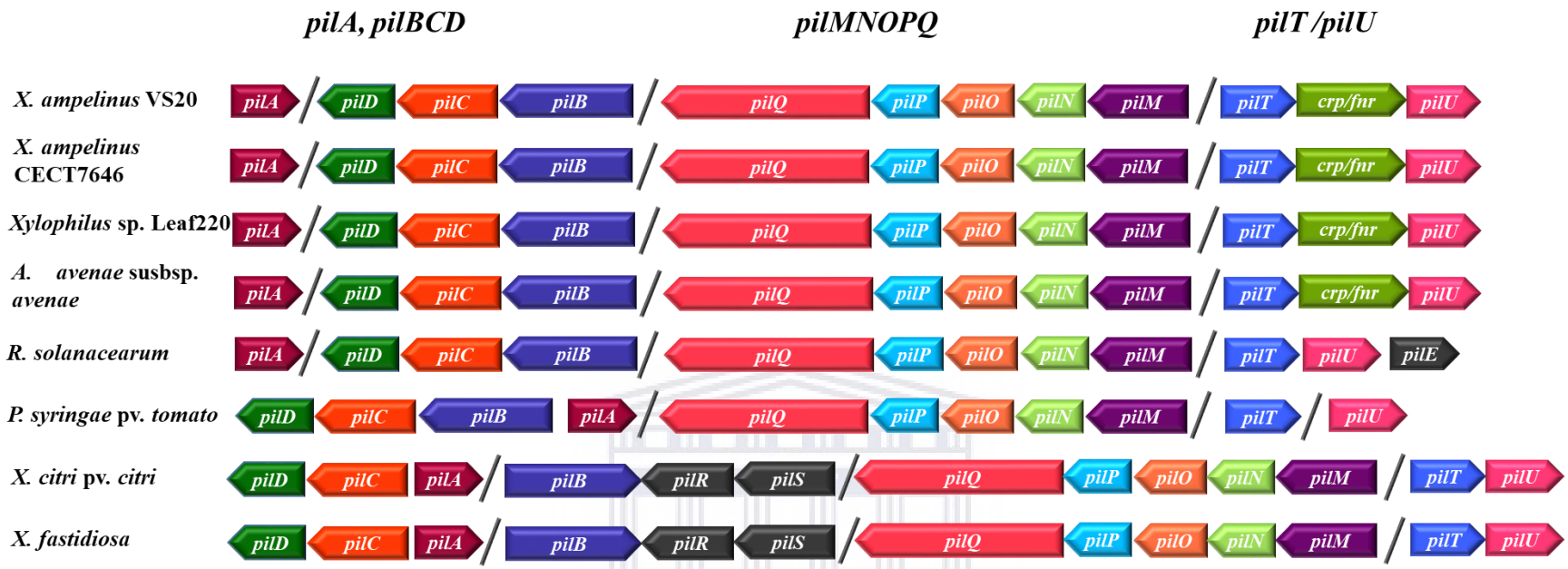

Figure 3.2. Organization of type IV pili (T4P) gene clusters of *Xylophilus ampelinus* isolates VS20 and CECT 7646, *Xylophilus* sp. Leaf220 and *Acidovorax avenae* subsp. *avenae* strain ATCC19860, *Ralstonia solanacearum*, *Pseudomonas syringae* pv. *tomato*, *Xanthomonas citri* pv. *citri*, and *Xylella fastidiosa*. Arrows indicate the direction of transcription of the genes from 5′ to 3′. The gene clusters are separated by the forward slash (/).

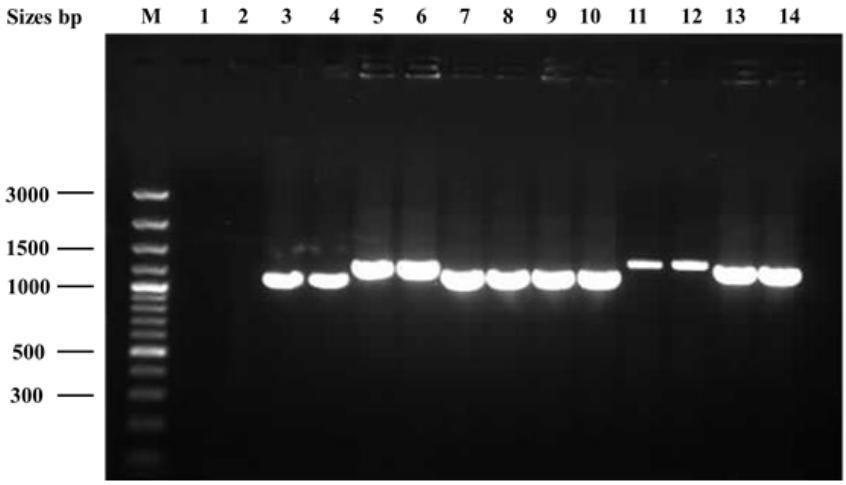

Figure 3.3. PCR confirmation of *X. ampelinus* T4P mutant strains. Agarose gel electrophoresis following PCR using primers amplifying the sequence upstream of the native promoter of each gene of interest together with the reverse sequence primer of the kanamycin gene. Lane M- GeneRuler 100 bp Plus DNA ladder (Thermo Scientific), lane 1- no template control, lane 2- polymerase negative control, lanes 3 and 4- *pilA*, lanes 5 and 6- *pilB*, lanes 7 and 8- *pilC*, lanes 9 and 10- *pilD*, lanes 11 and 12- *pilQ*, and lanes 13 and 14- *pilU*.

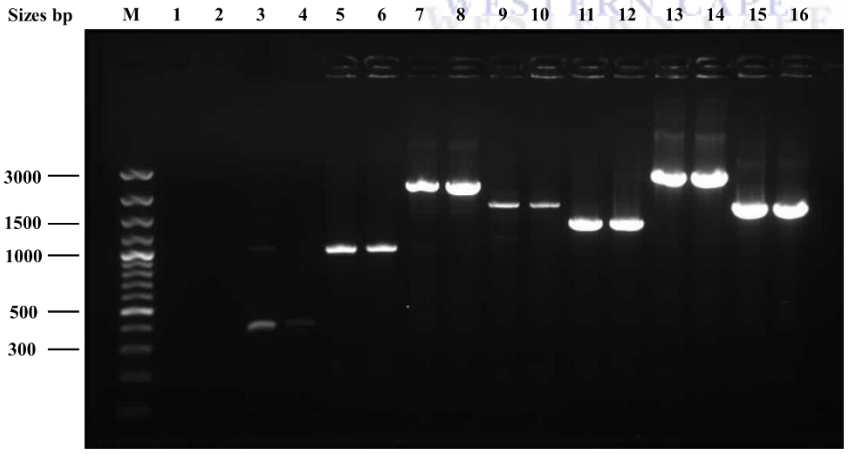

Figure 3.4. PCR confirmation of *X. ampelinus* T4P complemented strains. Agarose gel electrophoresis following PCR using pBBR1MCS5 *LacZ* operon primers. Lane M- GeneRuler 100 bp Plus DNA ladder, lane 1- no template control, lane 2- polymerase negative control, lanes 3 and 4- 366 bp LacZ operon amplicon lanes, 5 and 6- *pilA*, lanes 7 and 8- *pilB*, lanes 9 and 10- *pilC*, lanes 11 and 12- *pilD*, lanes 13 and 14- *pilQ*, and lanes 15 and 16- *pilU*.

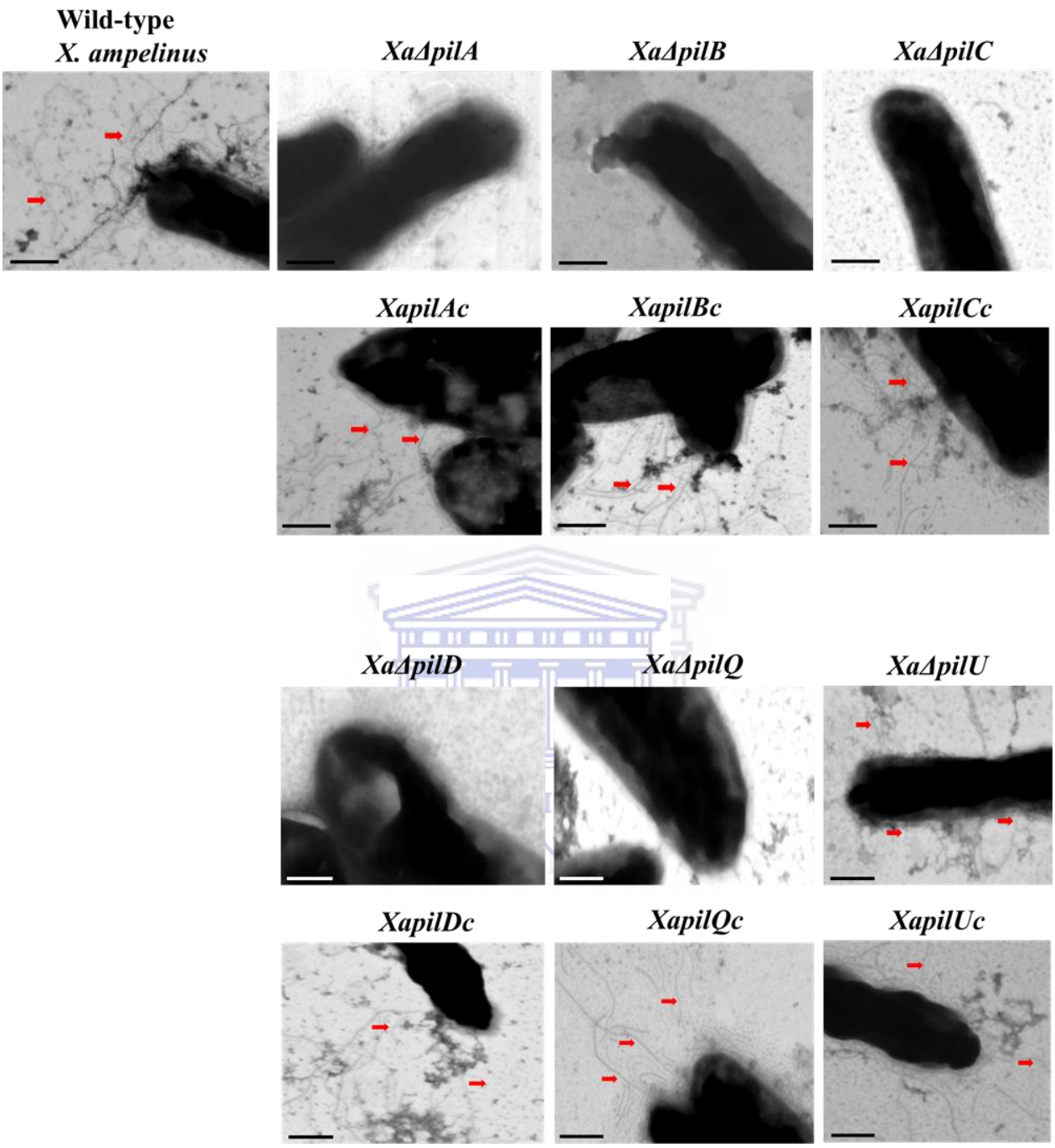

Figure 3.5. Scanning transmission electron microscopy (STEM) of wild-type *Xylophilus ampelinus* and type IV pili (T4P) gene deletion mutants (*XaΔpilA*, *XaΔpilB*, *XaΔpilC*, *XaΔpilD*, *XaΔpilQ*, and *XaΔpilU*) and their respective complemented strains (*XapilAc*, *XapilBc*, *XapilCc*, *XapilDc*, *XapilQc*, and *XapilUc*). The strains were grown on YPGA containing 1% agar for seven days before preparation for microscopy. Red arrows indicate T4P fibres. Scale bar =0.2 µm.

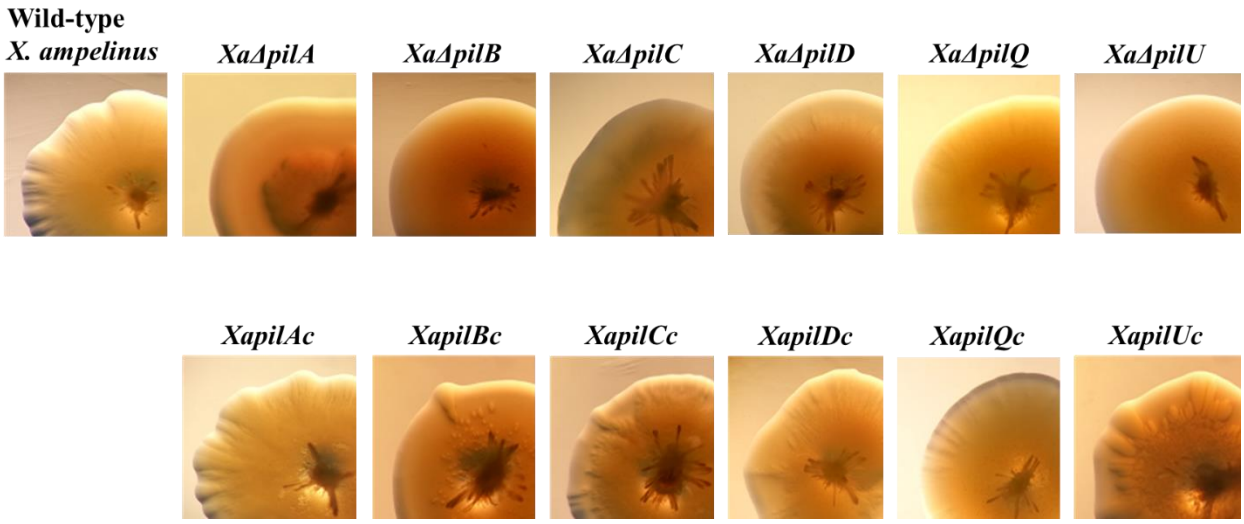

Figure 3.6. Twitching motility assay of wild-type *X. ampelinus*, and type IV pili (T4P) gene deletion mutants (*XaΔpilA*, *XaΔpilB*, *XaΔpilC*, *XaΔpilD*, *XaΔpilQ*, and *XapilU*) and their respective complemented strains (*XapilAc*, *XapilBc*, *XapilCc*, *XapilDc*, *XapilQc*, and *XapilUc*). The strains were grown on YPGA containing 1% agar for ten days before being photographed.

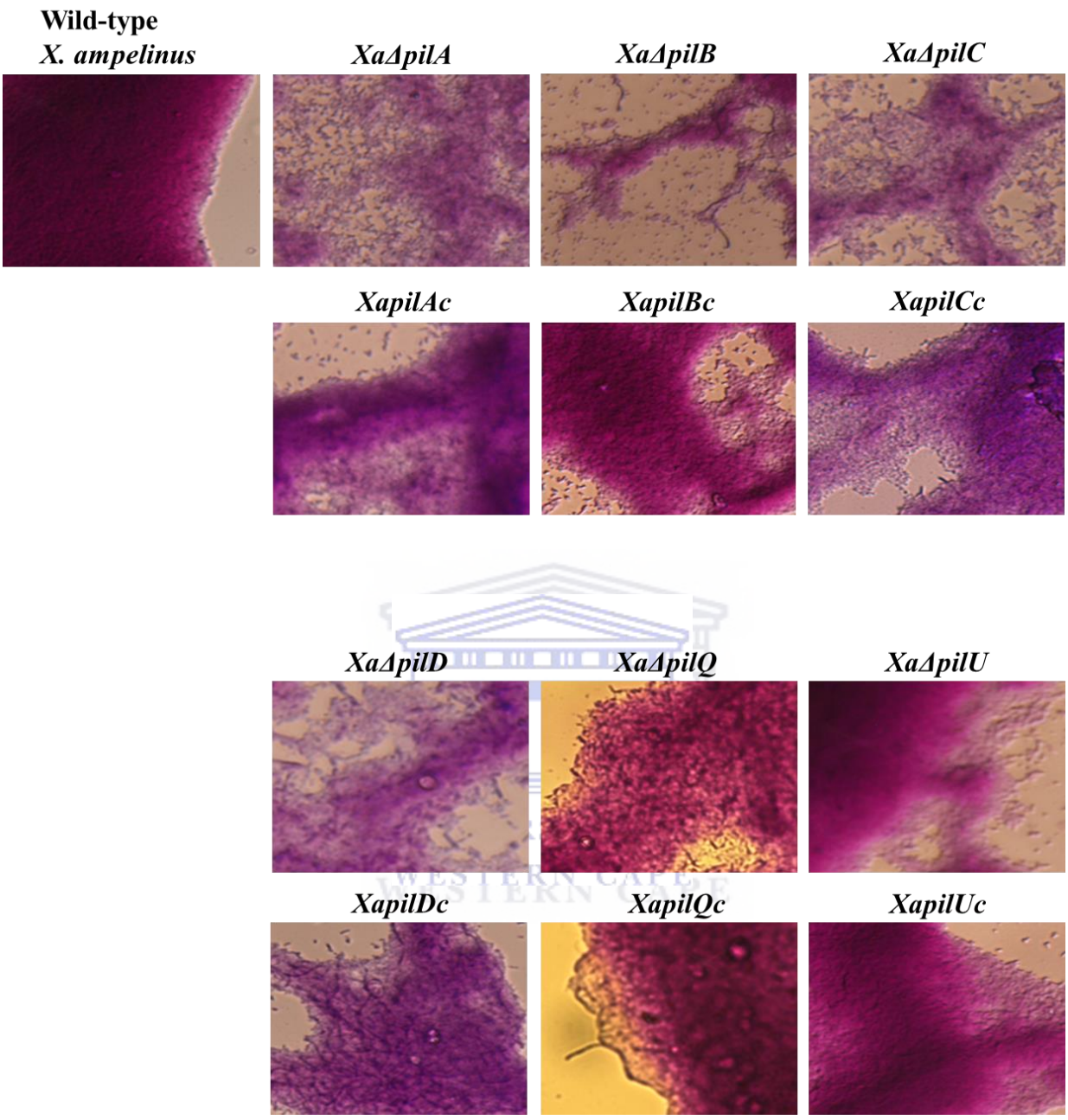

Figure 3.7. Biofilm formation of wild-type *Xylophilus ampelinus*, type IV pili (T4P) gene deletion mutants (*XaΔpilA*, *XaΔpilB*, *XaΔpilC*, *XaΔpilD*, *XaΔpilQ*, and *XaΔpilU*) and their respective complemented strains (*XapilAc*, *XapilBc*, *XapilCc*, *XapilDc*, *XapilQc*, and *XapilUc*) at the initial biofilm formation stage. The data shows light microscopy micrographs of the initial biofilms formed on glass slide coverslips taken at initial biofilm formation stage at day five post-inoculation. This experiment is representative of three independent experiments and all micrographs were taken under 100x magnification objective.

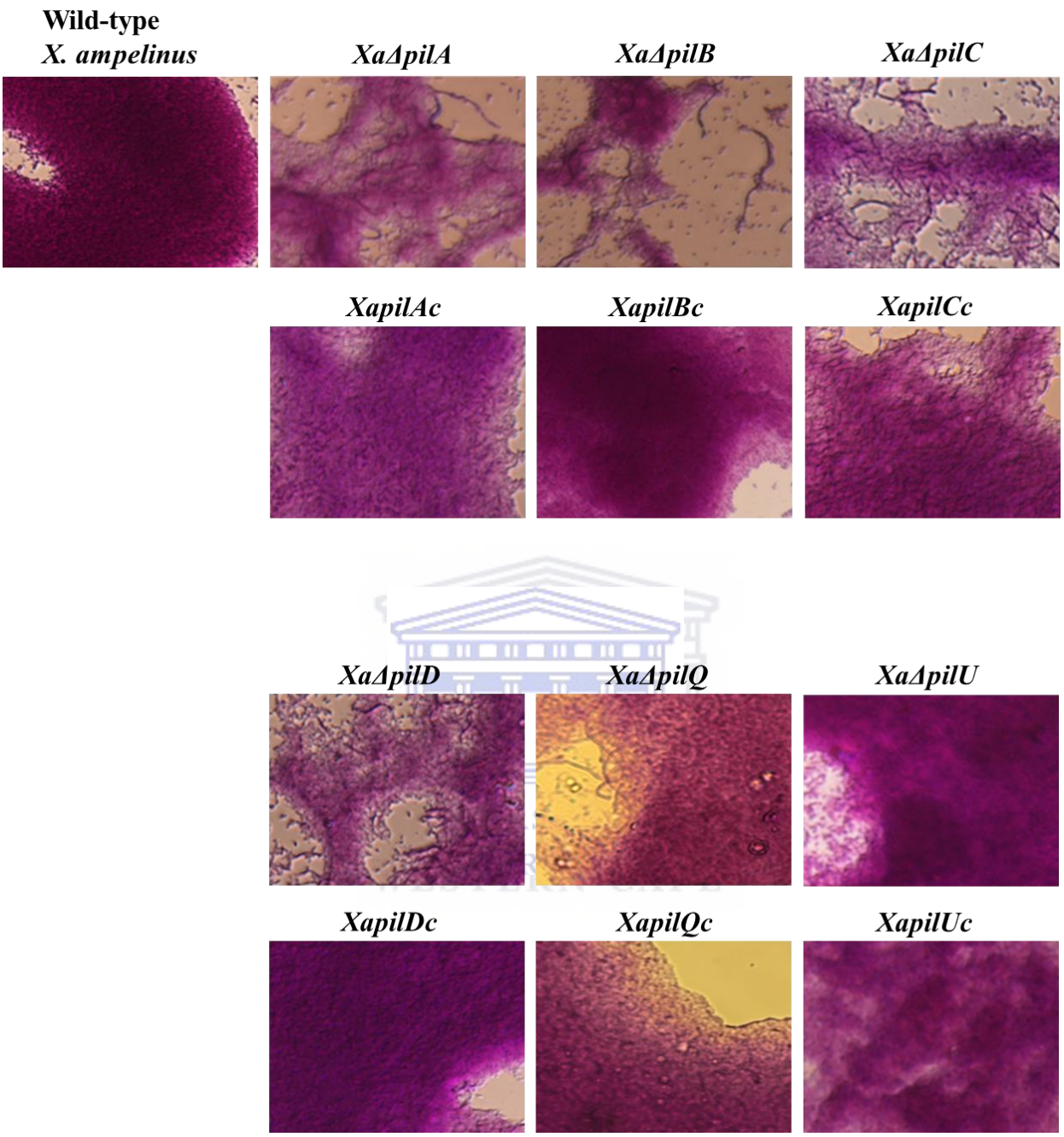

Figure 3.8. Biofilm formation of wild-type *Xylophilus ampelinus*, type IV pili (T4P) gene deletion mutants (*XaΔpilA*, *XaΔpilB*, *XaΔpilC*, *XaΔpilD*, *XaΔpilQ*, and *XaΔpilU*) and their respective complemented strains (*XapilAc*, *XapilBc*, *XapilCc*, *XapilDc*, *XapilQc*, and *XapilUc*) at the mature biofilm formation stage. The data shows light microscopy micrographs of the mature biofilms formed on the glass slide coverslips taken at the mature biofilm formation stage at day ten post-inoculation. This experiment is representative of three independent experiments and all micrographs were taken under 100x magnification objective.

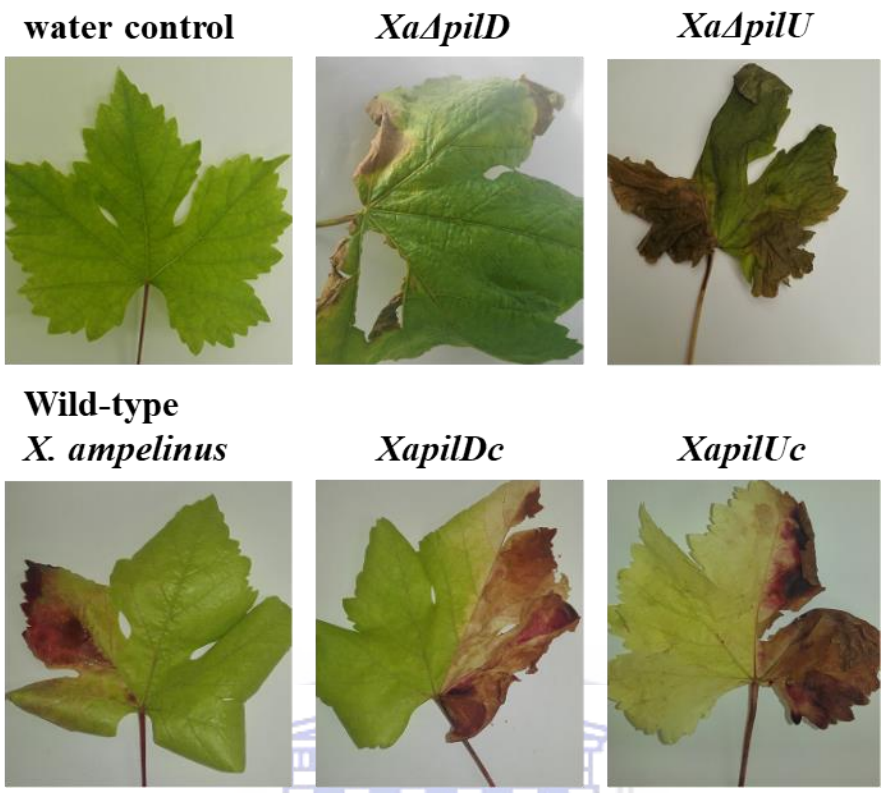

Figure 3.9. Bacterial blight symptoms on grapevine cultivar Redglobe leaves following inoculation with wild-type *X. ampelinus*, type IV pili (T4P) gene deletion mutants, *XaΔpilD* and *XaΔpilU* and their respective complemented strains, *XapilDc* and *XapilUc*. The photographs were taken at 12 weeks post-inoculation. Plants inoculated with the wild-type and complemented strains developed typical reddish-brown discolouration on leaves above the inoculation points.

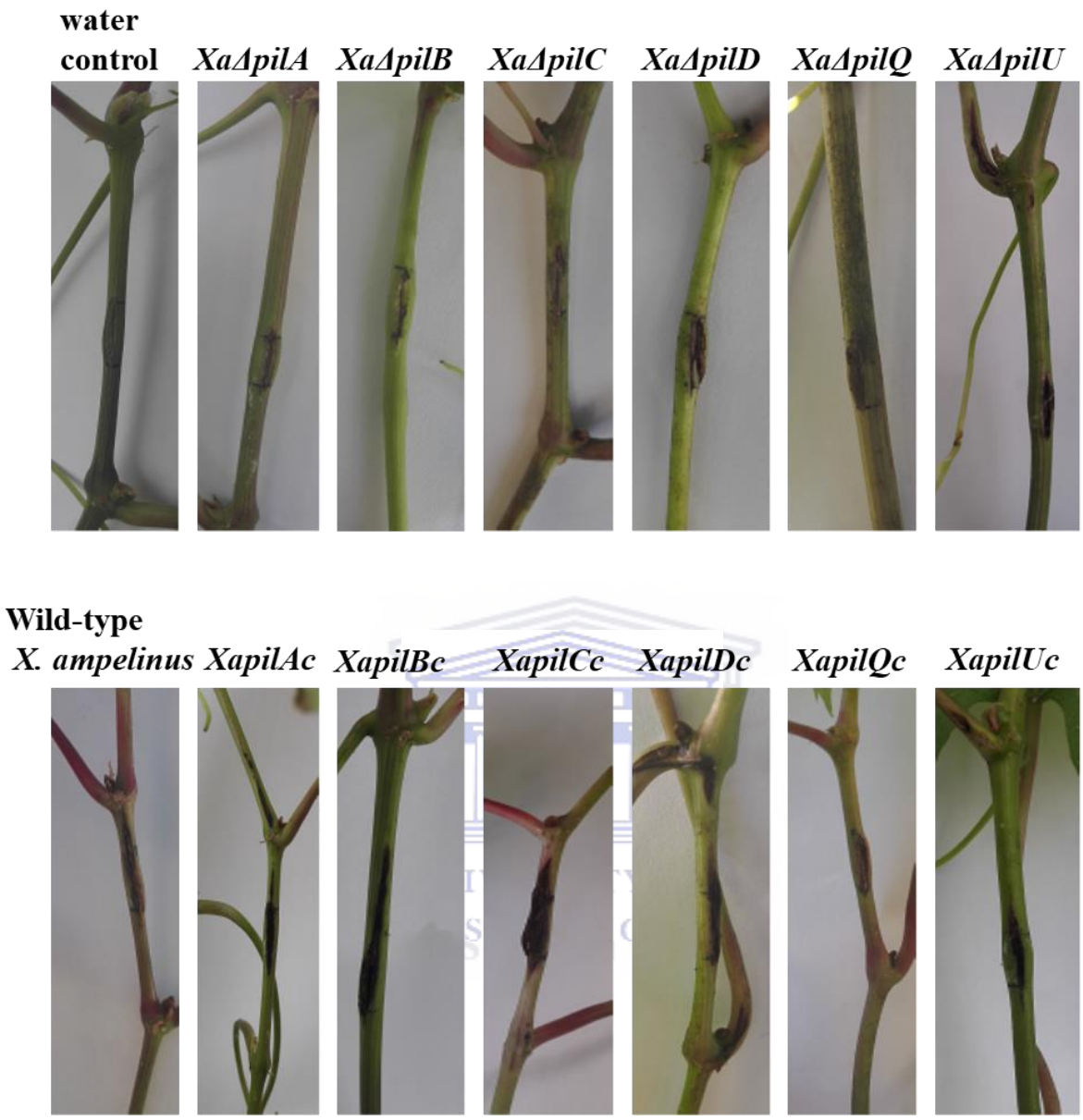

Figure 3.10. Bacterial blight symptoms on grapevine shoots inoculated with wild-type *X. ampelinus*, type IV pili (T4P) gene deletion mutants (*XaΔpilA*, *XaΔpilB*, *XaΔpilC*, *XaΔpilD*, *XaΔpilQ*, and *XaΔpilU*) and their respective complemented strains (*XapilAc*, *XapilBc*, *XapilCc*, *XapilDc*, *XapilQc*, and *XapilUc*). Photographs were taken at 12 weeks post-inoculation. Necrosis and cracks coupled with reddish-brown streaks characteristic of bacterial blight disease were observed on shoots inoculated with complemented strains and wild-type *X. ampelinus.*

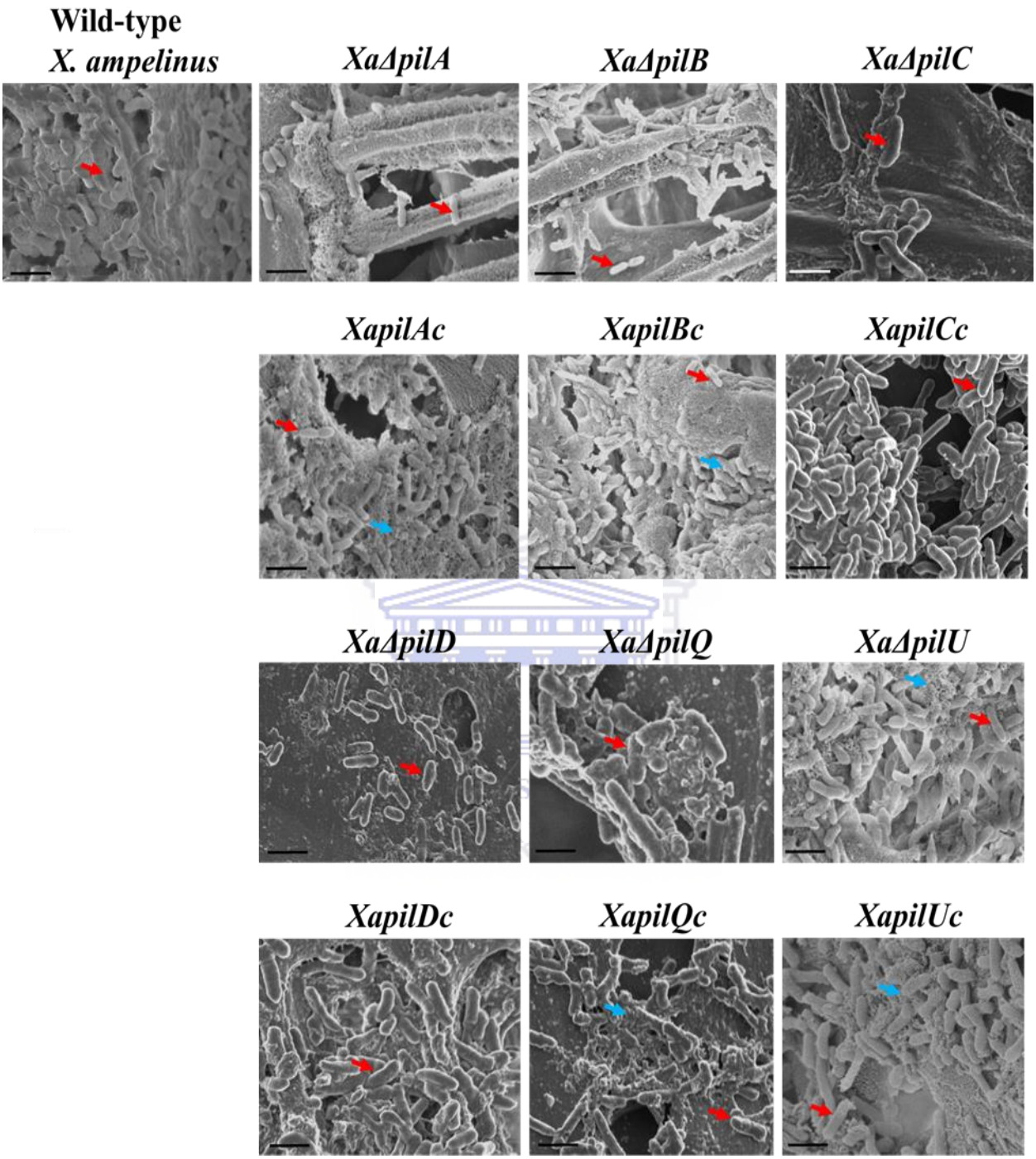

Figure 3.11. Scanning electron microscopy of a longitudinal section through the xylem vessels at the inoculation point of grapevine cultivar, Redglobe, at four weeks post-inoculation with wild-type *Xylophilus ampelinus* strain VS20, type IV pili (T4P) gene deletion mutants (*XaΔpilA*, *XaΔpilB*, *XaΔpilC*, *XaΔpilD*, *XaΔpilQ*, and *XaΔpilU*) and their respective complemented strains (*XapilAc*, *XapilBc*, *XapilCc*, *XapilDc*, *XapilQc*, and *XapilUc*). The red arrows indicate bacterial cells while the blue arrows indicate extracellular components of the biofilm matrix. Scale bars are 2 µm for all micrographs.

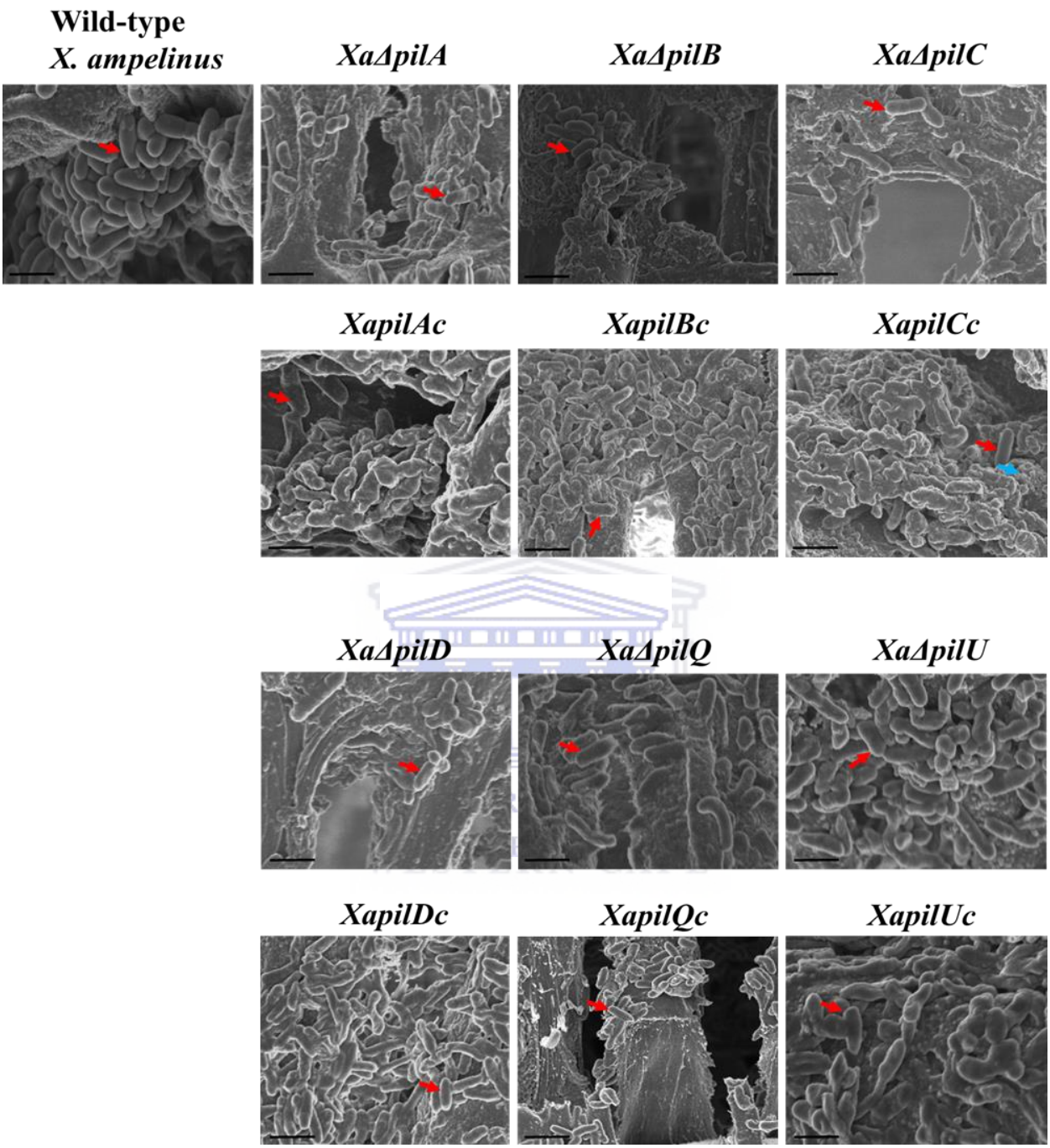

Figure 3.12. Scanning electron microscopy of a longitudinal section through the xylem vessels at the inoculation point of grapevine cultivar, Redglobe, at eight weeks post-inoculation with wild-type *Xylophilus ampelinus* strain VS20, type IV pili (T4P) gene deletion mutants (*XaΔpilA*, *XaΔpilB*, *XaΔpilC*, *XaΔpilD*, *XaΔpilQ*, and *XaΔpilU*) and their respective complemented strains (*XapilAc*, *XapilBc*, *XapilCc*, *XapilDc*, *XapilQc*, and *XapilUc*). The red arrows indicate bacterial cells while the blue arrows indicate extracellular components of the biofilm matrix. Scale bars are 2 µm for all micrographs.

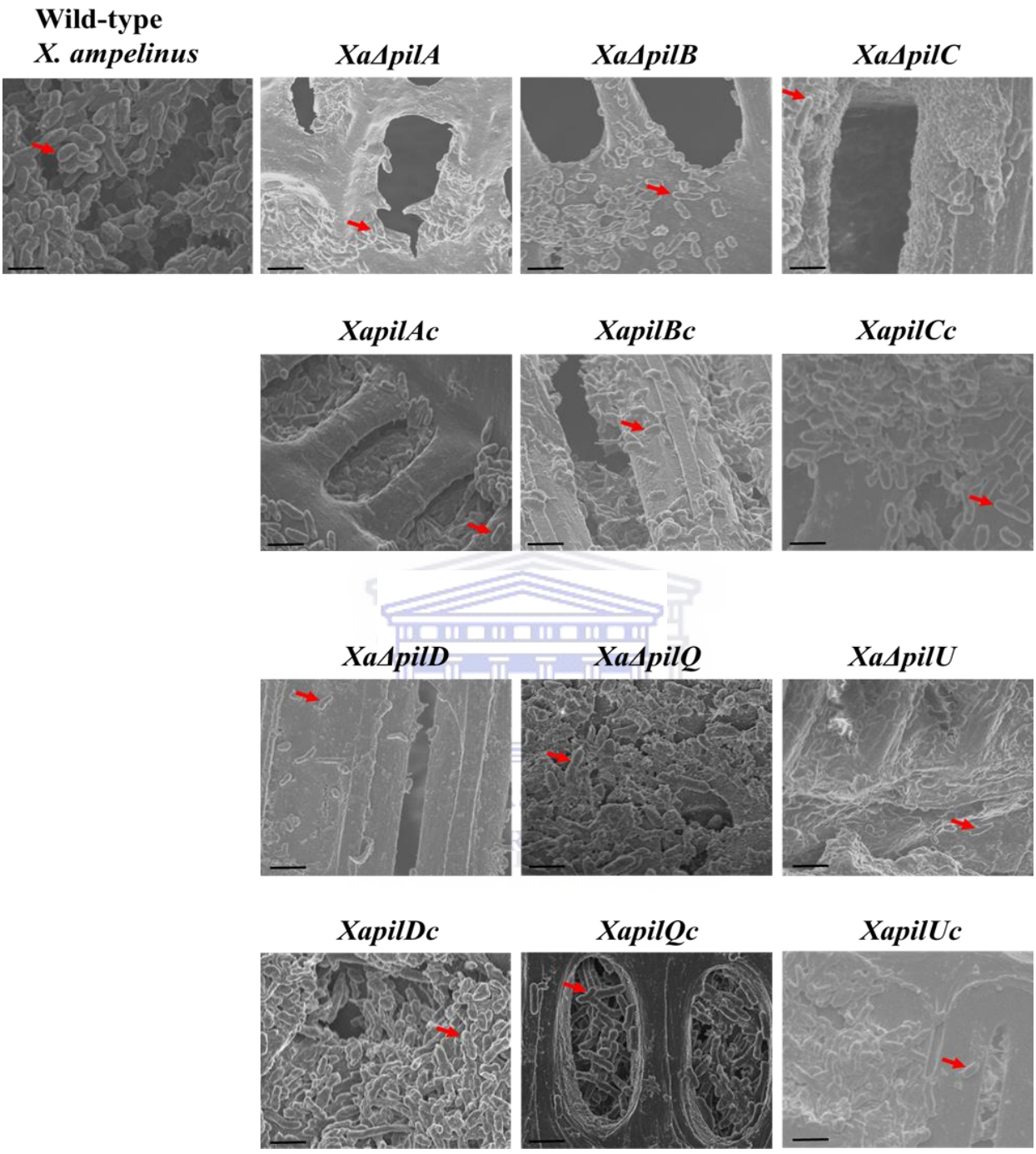

Figure 3.13. Scanning electron microscopy of the longitudinal section through the xylem vessels at the inoculation point of grapevine cultivar, Redglobe, at twelve weeks post-inoculation with wild-type *Xylophilus ampelinus* strain VS20, type IV pili (T4P) gene deletion mutants (*XaΔpilA*, *XaΔpilB*, *XaΔpilC*, *XaΔpilD*, *XaΔpilQ*, and *XaΔpilU*) and their respective complemented strains (*XapilAc*, *XapilBc*, *XapilCc*, *XapilDc*, *XapilQc*, and *XapilUc*). The red arrows indicate bacterial cells. Scale bars are 2 µm for all micrographs.

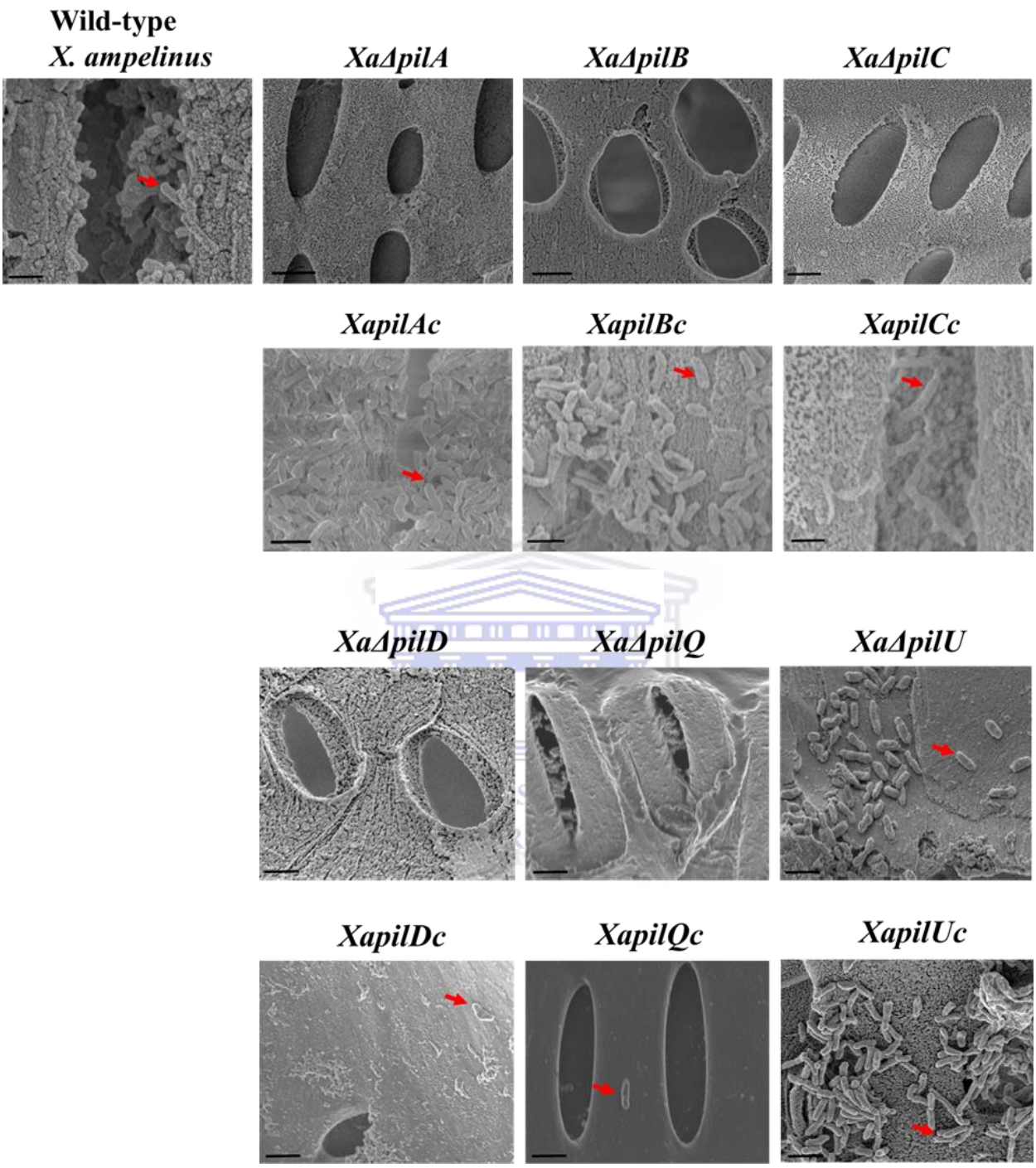

Figure 3.14. Scanning electron microscopy of a longitudinal section through the xylem vessels of grapevine cultivar, Redglobe, at 12 weeks post-inoculation with wild-type *Xylophilus ampelinus*, type IV pili (T4P) gene deletion mutants (*XaΔpilA*, *XaΔpilB*, *XaΔpilC*, *XaΔpilD*, *XaΔpilQ*, and *XaΔpilU*) and their respective complemented strains (*XapilAc*, *XapilBc*, *XapilCc*, *XapilDc*, *XapilQc*, and *XapilUc*). Colonization of the plant vessels at a five-centimetre distance above the point of inoculation. The red arrows indicate bacterial cells. Scale bars are 2 µm for all micrographs.

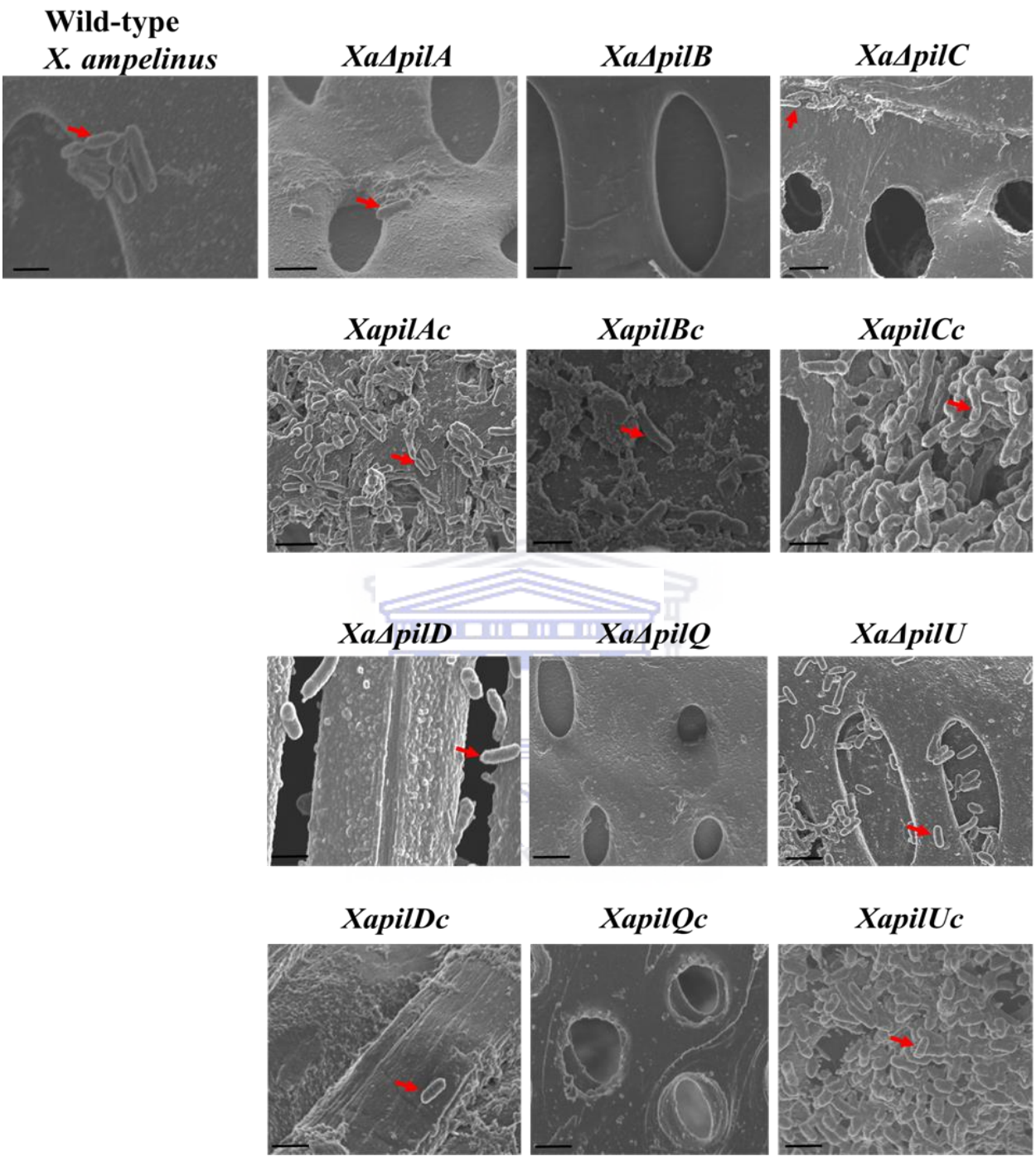

Figure 3.15. Scanning electron microscopy of a longitudinal section through the xylem vessels of grapevine cultivar, Redglobe, at 12 weeks post-inoculation with wild-type *Xylophilus ampelinus*, type IV pili (T4P) gene deletion mutants (*XaΔpilA*, *XaΔpilB*, *XaΔpilC*, *XaΔpilD*, *XaΔpilQ*, and *XaΔpilU*) and their respective complemented strains (*XapilAc*, *XapilBc*, *XapilCc*, *XapilDc*, *XapilQc*, and *XapilUc*). Colonization of the plant vessels at a five-centimetre distance below the point of inoculation. The red arrows indicate bacterial cells. Scale bars are 2 µm for all micrographs.

4. CHAPTER 4: Final Discussion

Biofilm formation accounts for a large part of the success of pathogens inside their hosts and is associated with persistent infections. The ability of a pathogen to form biofilms contributes to the pathogenesis of such pathogen. Because biofilm formation is a complex process with many factors surrounding its developmental processes, some of the major components of biofilm formation such as extra polymer substances (EPS), type IV pili (T4P), flagella, quorum sensing signaling, and c-di-GMP are required for the virulence of many pathogens, making biofilm formation a major determinant for pathogenicity.

This study sought to characterize and identify pathways required for biofilm formation and those required for the maintenance of the biofilm formed by *Xylophilus ampelinus* to improve our knowledge of how the bacteria interact, colonize, and cause disease inside its grapevine host. Because of the importance of T4P in micro-colony formation - a precursor step for biofilm formation and maturation - and surface motilities, the study also sought to characterize *X ampelinus* T4P and investigate the role of T4P-mediated twitching motility in host colonization and biofilm formation.

Biofilm formation is a complex and highly regulated process that involves attachment and interaction with surfaces, cell-to-cell communication, gene expression regulation, and extracellular polymer substance (EPS) production (Sauer and Camper 2001; Stoodley et al. 2002). Generally, the regulation of bacterial biofilm formation involves cell-to-cell communication, which leads to changes in gene expression and subsequently changes in behavioral traits of the cell/ community (Von Bodman et al. 2008). It is well known that the regulation of biofilms involves some of the pathways including the DSF-QS signaling pathway, c-di-GMP and small RNAs (Römling et al. 2013). Although this study did not identify the quorum sensing signals involved, there were some indications that the bacterium expresses the genes within the *rpf* (regulation of pathogenicity factors) cluster, responsible for DSF QS signal synthesis and perception. This observation leads to the strong belief that *X. ampelinus* may produce and respond to the DSF signal molecule that is known in other plant pathogens such as *Xylella* and *Xanthomonas* for the regulation of major processes such as biofilm formation, motility, EPS production, and virulence. The proteomic study identified two PAS-domain containing proteins that are well known for the diguanylate cyclase and phosphodiesterase activities required for c-di-GMP metabolism. Further bioinformatic

analysis of the PAS domain-containing proteins, showed that *X. ampelinus* is rich in these proteins, which appeared to be scattered throughout the genome. One of the PAS domain-containing proteins that was identified in this study was only induced during initial biofilms and could contribute to the transition of cells from planktonic to mature biofilms. The presence of the PAS, PAC, GGDEF, and EAL domains in this protein suggests that *X. ampelinus* regulates a number of processes through the modulation of c-di-GMP, including the suppression of flagella dependent motilities and production of EPS. The findings in this study also show suppression of a flagella biosynthesis protein in biofilms, indicating that flagella motilities are switched off when bacteria form biofilms, a process regulated through c-di-GMP modulation. In this study, similar to the *Xanthomonas axonopodis* pv. *citri* biofilm proteome analysis study (Zimaro et al. 2013), none of the T4P complex proteins were identified, even though they may have been expressed under the conditions tested using the proteome analysis approach. This may be due to the limitations posed by the methods used in this study that only allows the analysis of proteins within the immobilized pH gradient (IPG) strip with a pH range of 4 to 7 compared to the methods using IPG strips with a 3 to 10 pH range or in-solution digest procedure.

In this study, two proteins responsible for EPS breakdown were suppressed and reduced, respectively i.e. the cellulose 1,4-beta-cellobiosidase required for the breakdown of cellulose, a biofilm matrix component EPS, and polyribonucleotide nucleotidyltransferase required for negative regulation of poly-N-acetylglucosamine, a major polysaccharide also forming part of the biofilm matrix. These findings suggest that *X. ampelinus* requires cellulose and PNAG as major biofilm matrix components, both of which are c-di-GMP targets, indicating that the production of EPS in *X. ampelinus* biofilms is dependent upon the c-di-GMP regulatory pathway. Therefore, the presence of the diguanylate cyclases (DGCs) and phosphodiesterases (PDEs) in *X. ampelinus* and the induction of the two PAS domains during biofilm formation by this bacterium shows that *X. ampelinus* requires c-di-GMP to regulate biofilm formation.

Here also in this study, the role of type IV pili (T4P), an organelle required for surface twitching motility that drives micro-colony formation during biofilm formation was investigated through gene mutation and complementation. The study results show that *X. ampelinus* requires the major pilin subunit, pre-pilin peptidase enzyme, extension ATPase, outer-membrane secretin, and a platform protein for the synthesis and expression of the T4P and failure of these gene mutants to

express T4P shows that these genes are indispensable for the T4P assembly. The function of the PilU protein has remained an enigma in many bacterial species; however, the results obtained in this study showed that the retraction ATPase and twitching motility protein PilU is not required for the biosynthesis of T4P but for twitching motility function. Therefore, twitching motility of *X. ampelinus* is dependent upon the properly assembled and fully functional T4P because all the T4P-deficient strains and the T4P-expressing strain with defects in the retraction ATPase gene were unable to twitch under inducing conditions. When tested for the role of T4P in biofilm development *in vitro*, pili-deficient and twitch-minus strains (except *pilQ*) were also impaired in their ability to form biofilms but formed weakened structures of cell aggregates that failed to develop into a mature biofilm signifying the importance of the T4P-mediated micro-colony development in biofilm maturation. Similarly, further analysis of the role of T4P in biofilm formation *in planta* also showed that T4P plays a role in biofilm formation and colonization of plant surfaces inside the plants. Failure of the T4P-deficient mutant strains (except *pilQ*) to form biofilms at the point of inoculation showed that T4P play a crucial role in the establishment of the pathogen in the host during the early stages of infection. On the other hand, biofilms formed by the *pilU* mutant at the point of inoculation signifies that the adhesive nature of the T4P facilitated attachment and the lack of the retraction function in this mutant allowed the irreversible attachment of the cells at the point of inoculation, thus limiting the movement of the cells.

Through SEM analysis of the plant vasculature, it was shown that *X. ampelinus* requires T4P to migrate inside the plants as *pilA*, *pilB*, *pilC*, and *pilQ* mutants deficient in T4P-expression were unable to move from the point of inoculation to distal parts of the plants. Furthermore, the full virulence exhibited by the wild-type and complemented strains that induced symptom expression and formed biofilms at the distal parts away from the inoculation point, indicates that T4P-dependent twitching motility facilitates biofilm formation in plants, which thereby, contributes to full virulence. This concept was further confirmed by the failure of the *pilU* mutant cells to form micro-colonies and biofilms at a 5 cm distance away from the inoculation point even though the cells were present at this point (as detected by nPCR and SEM). Therefore, the observed role of T4P in biofilm development inside plants and the reduced virulence exhibited by all the T4P mutants indicate that T4P are required for *in planta* biofilm formation and surface colonization

through twitching motility and ultimately T4P are required for the virulence of *X. ampelinus* in grapevine host.

The results obtained in this study contribute to the understanding of *X. ampelinus* pathogenicity factors. Understanding of the biofilm formation process is a crucial step to elucidating the pathways involved in the adaptation, survival, and infection process by this pathogen.

Future perspectives

This study identified a number of proteins with possible roles in the regulation of biofilm formation by *X. ampelinus*, and expansion from this work is required to acquire the definite functions of such regulatory proteins. Primarily, functional characterization of the the PAS domain containing protein identified in this study which has proposed functions during the transition of bacteria from planktonic to biofilm growth is required. Furthermore, this study identified a protein belonging to the clp protein family (ClpP) with an opposing role in the regulation of biofilm formation and EPS production through the modulation of c-di-GMP levels in the cells compared to the other ATPases in this family. Further analysis of the proteins in this family and their functions in controlling c-di-GMP concentration in relation to biofilm formation is also required. Another pathway associated with the regulation of pathogenicity factors (*rpf*) gene cluster, not previously mentioned in *X. ampelinus*, was identified in this study, however, the function of these genes in the virulence of *X. ampelinus* is still unkown and their relation to DSF quorum sensing pathway is still not yet known. Unpacking the functions of these pathways may bring us a step closer to understanding the pathogenicity factors required for the virulence of *X. ampelinus* in grapevine plants and may provide possible strategies of combating the disease.

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