

Secretome analyses of selected *Pectobacterium brasiliense* 1692 secretion systems

By

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DECLARATION

I, Silindile Maphosa, hereby declare that the thesis is submitted for the Ph.D. degree in Biotechnology at the University of Pretoria. This is my own work and has not previously been submitted by me for a degree at this or any other institution.

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ACKNOWLEDGEMENTS

I am grateful to God, who sees everything and knows every plan He has for me. I am alive and well post the terrifying COVID-19 pandemic.

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SUMMARY

Soft Rot *Pectobacteriaceae* (SRP) is a family of Gram-negative bacteria (GNB) that causes damage to crop and ornamental plants. *Pectobacterium brasiliense* 1692 (*Pbr*1692) is among the most devastating *Pectobacterium* species. Therefore, it is important to understand the biology of this strain, including strategies used by SRP to adapt to changing environments, colonise susceptible hosts, and persist in the environment. Therefore, the contribution to the knowledge pool about *Pbr*1692 through this study entailed the determination of the secretome of two secretion systems, outer membrane vesicles (OMVs) and the type 6 secretion system (T6SS). These transport diverse proteins for multiple roles into the environment and target cells. In this study, their secretome profiles suggest that OMVs and the T6SS potentially contribute to the ecological success of *Pbr*1692.

Chapter One: Secretion systems form part of the mechanisms underlying bacteria-bacteria interactions, pathogenesis, and disease development in eukaryotic hosts. This chapter reviews the literature on Gram-negative bacteria secretion systems, the dialogues that possibly exist between them, and the implications of their interactions. Several cases of inter-secretion system functioning in bacterial rivalry, virulence, and survival, *inter alia*, are highlighted. The review also focuses on the importance of studying secretion systems as inter-dependent entities to unlock some fundamentals into the biology and functions of bacteria. Furthermore, it delves into the possible implications of the dialogues for bacteria in their natural niches.

Chapter Two: In this chapter, vesicle-associated proteins were identified from isolated *Pbr*1692 OMVs. From these OMVs, 129 proteins in varied levels of abundance were functionally characterised using sequence analyses following identification by mass spectrometry. Functional annotation revealed that OMVs harbour and associate with cargo including proteins with phospholipase A1 or A2, polygalacturonase, peptidase, and cellulase activity that serve diverse roles beneficial to the phytopathogen, *Pbr*1692. Some of the OMV cargo proteins were predicted T6SS and T2SS substrates. Substrate signal peptide prediction analyses indicated possible signal sequence independent secretion by the OMVs. Using different phenotypic assays, we verified some of the *in silico* predicted functions. Protease activity of OMVs was shown using

gelatine zymography. Competition assays showed that protein cargo of *Pbr1692* OMV is involved in competitive fitness against *Dickeya dadantii*. Virulence on susceptible potato variety *Solanum tuberosum* cultivar (cv.) Mondial tubers was also observed. Furthermore, pathogen associated molecular pattern (PAMP) triggered immunity was observed by elicitation of a hypersensitive response (HR) in *Nicotiana benthamiana* leaves.

Chapter Three: The focus of this chapter was to identify the role of the T6SS in *Pbr1692* protein secretion and its role in the potential interplay by implementing a combination of computational and proteomic analyses. These revealed possible interdependence between other secretion systems and the T6SS. *In silico* prediction using the Bastion6 and BastionX computational tools revealed that there is potential substrate co-secretion between the T6SS and T1SS-T4SSs. Among the predicted shared substrates were the T2SS substrates, including Avirulence protein L (AvrL), which was also found as an OMV cargo in Chapter Two of this thesis. Therefore, the predictions suggested that the T6SS of *Pbr1692* might assist in the secretion of T2SS substrates. To validate the *in silico* predictions, the *Pbr1692* wild type (T6SS-active strain) and *tssBC* sheath mutant (T6SS-inactive strain) secretomes were compared to the predicted substrates. To this end, *Pbr1692* strains were grown in virulence gene-inducing media to induce the T6SS and other virulence genes. Using mass spectrometry, 446 secreted proteins were identified from T6SS-inactive and T6SS-active cell-free supernatants. Comparing the T6SS-inactive and T6SS-active strain secretomes revealed 19 proteins to be secreted only by the wild type strain. Thirty proteins were differentially secreted by the T6SS-inactive and active strain. In total, 49 proteins were identified as T6SS associated proteins. Comparison of the *in vitro* T6SS substrates to predicted T1SS-T6SS substrates validated 25 candidates predicted via the computational pipeline. Overall, the results imply interplay between secretion systems and the T6SS via an unknown mechanism.

Chapter Four: In Chapters Two and Three, Avirulence L (AvrL), an SRP T2SS substrate was identified as an OMV substrate and predicted as a T6SS substrate, respectively. In the current chapter, computational characterisation of AvrL was carried out to understand the protein that is predicted to have three secretion systems dedicated toward its secretion. This is a developmental chapter whose main goal is to

build a solid basis for future work. Evolutionary analyses revealed that over 100 SRP encode *avrL* homologs. Further analyses predicted that AvrL contains a putative IpxD domain potentially involved in lipid A biosynthesis and a zinc-binding metallopeptidase domain involved in plant cell wall degradation. In addition, seven intrinsically disordered regions were identified in this protein which are predicted to form part of a host protein mimicry strategy. In terms of interacting partners, AvrL seems to be functionally linked to ten T2SS substrates, which further supports its association with the T2SS. A molecular interaction map of the AvrL with the model plant *Arabidopsis thaliana* protein-protein interaction network revealed 152 candidate host protein targets. A total of 150 *Pbr1692* host potato (*Solanum tuberosum*) proteins were identified as *A. thaliana* interactor functional homologs. Nineteen differentially expressed genes, mostly transcription factors were identified to code for AvrL potential targets in *Solanum tuberosum* cv. Valor when infected by *Pbr1692*. The results of this chapter imply that AvrL potentially has spatial roles in *Pbr1692* including host defence response manipulation for pathogen benefit.

Altogether, the study concluded that GNB interactively use their secretion systems to achieve the secretion of a wide range of proteins that are crucial for the biology of *Pbr1692*. However, we recommend verification of these implications by future work.

THESIS OUTPUTS

Scientific publications:

Maphosa Silindile, and Lucy Novungayo Moleleki. Isolation and characterisation of outer membrane vesicles of *Pectobacterium brasiliense* 1692. *Microorganisms* 9.9 (2021): 1918 (First author).

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2021 Identification and functional characterisation of *Pectobacterium* spp. outer membrane vesicles – World Microbe Forum (WMF2021, virtual meeting).

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LIST OF ABBREVIATIONS

%	Percentage
±	Plus, or minus
®	Registered sign
°C	Degrees Celsius
µg	Microgram
µg/mL	Microgram per millilitre
µl	Microlitre
µm	Micromolar
2-DE	Two-dimensional gel electrophoresis
<i>A. thaliana</i>	<i>Arabidopsis thaliana</i>
ACD	Actin-crosslinking domain
ACN	Acetonitrile
Al	Aliphatic
ARO	Antibiotic resistance ontology
Avr	Avirulence
AvrL	Avirulence protein L
BGI	Beijing genomics institute
BGM	Biochemistry, genetics & microbiology
Bhlh	Basic helix-loop-helix
BlastP	Protein basic local alignment search tool

BP	Biological process
CAMP	Cationic antimicrobial peptide
CARD	Comprehensive Antibiotic Resistance Database
CAZymes	Carbohydrate-active enzymes
CC	Cellular component
CDD	Conserved Domain Database
CDI	Contact-dependent Inhibition
CIS	Contractile injection systems
COGs	Clusters of orthologous groups
CuS NPs	Copper sulphide nanoparticles
Cv	Cultivar
<i>D. dadantii</i>	<i>Dickeya dadantii</i>
Da	Dalton
DAVID	Database for Annotation, Visualisation, and Integrated Discovery
DDA	Data dependent acquisition
DEGs	Differentially expressed genes
DEPs	Differentially expressed proteins
DNA	Deoxyribonucleic acid
dOMVs	Denatured outer membrane vesicles
DspE/A	Avirulence effector proteins in Soft Rot <i>Pectobacteriaceae</i>
DSPs	Differentially secreted proteins

DTT	Dithiothreitol
EC	Extinction coefficient
EDTA	Ethylenediaminetetraacetic acid
EF-Tu	Elongation factor-Tu
ELMs	Eukaryotic linear motifs
EOMVs	Explosive outer membrane vesicles
EPS	Extracellular polysaccharide
ESI-MS/MS	Electrospray ionisation mass spectrometry
ETEC	Enterotoxigenic <i>Escherichia coli</i>
FA	Formic acid
FABI	Forestry and agricultural biotechnology institute
FE-SEM	Zeiss crossbeam 540 field emission-scanning electron microscope
FIX	Found in type six
g/L	Grams per litre
GNB	Gram-negative bacteria
GNB	Gram-positive bacteria
GO	Gene ontology
GRAVY	Grand average of hydropathicities
h	Hour
Hcp	Hemolysin coregulated protein
HGT	Horizontal gene transfer

HMDS	Hexamethyldisilazane
HMM	Hidden Markov Model
HPI	Host-pathogen interaction
hpi	Hours post infection
HPLC	High performance liquid chromatography
HR	Hypersensitive response
<i>hrc</i>	Hypersensitive response conserved genes
<i>hrp</i>	Hypersensitive response and pathogenicity
HSI-2	Hcp secretion island-2
IAM	Iodoacetamide
iBAQ	Intensity based absolute protein quantification
ID	Identity
IDRs	Intrinsically disordered regions
II	Instability index
InterSPPI	Inter-Species Protein–Protein Interaction predictor
KDa	Kilodaltons
KEGG	Kyoto encyclopaedia of genes and genomes
L	Ladder
LB	Luria Bertani
LFQ	Label free quantitative
Log	Logarithm

LPS	Lipopolysaccharides
M	Marker
M	Molar
m/z	Mass to charge ratio
MAMPs	Microbe associated molecular patterns
MF	Molecular function
mg/mL	Milligrams per millilitre
min	Minute
MIX	Marker for type six
mL	Millilitre
mM	Millimolar
Mol/L	Moles per litre
MoRFs	Molecular recognition features
MS/MS	Tandem mass spectrometry
MSI	Mass spectrometry imaging
MV	Membrane vesicles
MW	Molecular weight
MWCO	Molecular weight cut-off
<i>N. benthamiana</i>	<i>Nicotiana benthamiana</i>
NCBI	National Centre for Biotechnology Information
ng	Nanogram

nL/min	Normal litres per minute
Nm	Nanometre
NTA	Nanoparticle Tracking Analysis
OD	Optical density
O-IMVs	Outer-inner membrane vesicles
Omp	Outer membrane protein
OMV	Outer membrane vesicles
PAAR	Proline alanine alanine arginine
PAMPS	Pathogen associated molecular patterns
<i>Pbr</i>	<i>Pectobacterium brasiliense</i>
<i>Pbr1692</i>	<i>Pectobacterium brasiliense</i> 1692
PBS	phosphate buffered saline
PCR	Polymerase chain reaction
PCWDE	Plant cell wall degrading enzyme
peh	Polygalacturonase
pel	Pectate lyase
PGSC	Potato Genome Sequencing Consortium
pH	Logarithm of the reciprocal of hydrogen-ion concentration
pI	Theoretical isoelectric point
PlantTFDB	Plant Transcription Factor DataBase
PONDR® VL-XT	Predictor of Natural Disordered Regions predictor

PPI	Protein-protein interactions
PRR	Plant recognition receptors
Prt	Protease
Psi	Pounds per square inch
PSIC	Position-specific independent count
PSM-level FDR	Peptide to spectrum match false discovery rate
PSSM	Position specific scoring matrix
Pv	Pathovar
QS	Quorum sensing
R	Resistance
RNA	Ribonucleic acid
RNA-Seq	RNA sequencing
RND	Resistance nodulation division
ROS	Reactive oxygen species
rpm	Revolutions per minute
s	Second
SCF	Skp1-cul1-f-box protein
SDS PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
SDT	Sequence Demarcation Tool
SEM	Scanning electron microscopy
SIAS	Sequence Identities and similarities

SLiMs	Short linear motifs
SN	Supernatant
SP	Signal peptide
spp	Species
SRP	Soft Rot <i>Pectobacteriaceae</i>
SS	Secretion system
<i>S. tuberosum</i>	<i>Solanum tuberosum</i>
subsp.	Subspecies
svx	Secreted virulence factor from <i>Xanthomonas</i>
T1SS	Type 1 secretion system
T2SS	Type 2 secretion system
T3SE	Type 3 secreted effector
T3SS	Type 3 secretion system
T4SS	Type 4 secretion system
T5SS	Type 5 secretion system
T6SE	Type 6 secreted effector
T6SS	Type 6 secretion system
T7SS	Type 7 secretion system
T8SS	Type 8 secretion system
T9SS	Type 9 secretion system
T10SS	Type 10 secretion system

T11SS	Type 11 secretion system
TCP	Teosinte branched1, cycloidea, pcf1
TEM	Transmission electron microscopy
TF	Transcription factor
TssBC	Type 6 secretion contractile sheath proteins
TXSSs	Type X secretion system
v	Version
v/v	Volume per volume
VF	Virulence factor
VgrG	Valine glycine repeat G
w/v	Weight per volume
WT	Wild type
α	Alpha
β	Beta

LIST OF FIGURES

Figure 1.1: Secretion systems (type 1-type 6 secretion system) of Gram-negative bacteria and available evidence of crosstalk with other secretion systems. The figure shows the six secretion systems of GNB (T1SS-T6SS) and the dialogs they are involved in with other secretion systems. The dotted lines indicate interaction at both substrate and system levels, while solid lines indicate interaction at either substrate or system level.

Figure 2.1: Identification of outer membrane vesicles (OMVs) produced by *Pectobacterium brasiliense* 1692. (A, B) In the scanning electron microscope images, gold arrows indicate OMVs of cells and OMV aggregates in the exponential growth phase. The white arrows show vesiculation “hot spots”. (C) The image of cells in stationary growth shows OMVs at the surface of the bacteria indicated by white arrows. Gold arrows indicate OMV aggregates and “hot spots.” (D) The image shows OMVs embedded in the biofilm matrix-like backbone. Scale bars = 1 μ m.

Figure 2.2: Transmission electron microscope identification of budding vesicles on *Pectobacterium brasiliense* 1692 cells and isolated outer membrane vesicles (OMVs) size and distribution. (A) The arrow indicates an OMV budding from a *Pbr*1692 cell. Scale bar = 200 nm. (B) Nanoparticle tracking analysis size distribution and quantification of isolated OMV assessment shows the distribution of *Pbr*1692 OMVs with an average size of 173.7 ± 1.9 nm isolated in the stationary growth phase.

Figure 2.3: Transmission electron microscopy (TEM) of outer membrane vesicles (OMVs) isolated from *Pectobacterium brasiliense* 1692 in the stationary growth phase. (A) The TEM image shows OMVs of different sizes. (B) The image shows OMV aggregates (top) and the OMV chain-like morphology (bottom). (C) The dotted circles indicate a single-membrane vesicle and a double-membrane outer inner membrane vesicle with a halo around. Scale bars = 200 nm.

Figure 2.4: Shared and exclusive outer membrane vesicle (OMV) associated proteins of biological replicates and shared protein localisation predictions. (A) The Venn diagram indicates shared and exclusive proteins of the three biological replicate OMVs isolated from *Pectobacterium brasiliense* 1692 cultures. Eighty proteins were found

present in all three biological replicates. **(B)** The pie chart shows the percentages of shared OMV proteins' predicted localisations.

Figure 2.5: Outer membrane vesicle protein molecular functions and protein clusters of orthologous groups classification. **(A)**. The graph shows the molecular ontology entries against the number of associated proteins. **(B)** The chart shows protein functional group categories and the corresponding number of proteins associated with each cluster.

Figure 2.6: Outer membrane vesicle (OMV) proteins associated with virulence, carbohydrate metabolism, stress resistance, and competition. The graph shows the number of OMV virulence factors, carbohydrate-active enzymes, antibiotic resistance agents, and type 6 secreted effectors.

Figure 2.7: Virulence activities of *Pectobacterium brasiliense* 1692 outer membrane vesicles (OMVs). **(A)** OMV protease activity. A 12% sodium dodecyl-sulfate polyacrylamide gel electrophoresis zymogram gel shows *Pbr*1692 OMV digestion of gelatine via protease activity. Gelatinase digestion by the OMVs is demonstrated by the white band clearing zone corresponding to a ~55 kDa protein by visualisation using Coomassie Brilliant blue staining. Denatured OMVs were used as a negative control. M = marker; dOMVs = denatured OMVs. **(B)** Soft rot of potato tubers by *Pbr*1692 OMVs. The figure shows OMV-associated maceration of potato tuber tissue three days post-inoculation. The cell-free supernatant was used as the positive control, while PBS was used as a negative control. The results show the means of two independent experiments. Error bars represent the standard deviations from the data. Differences between the effect of the control and OMVs were determined to be statistically significant with $p < 0.01$. **(C)** OMVs elicited a hypersensitive response in three-week-old seedlings of *Nicotiana benthamiana* 24 hpi. The OMV wash step supernatant (SN) and cell-free SN were used as controls.

Figure 2.8: Antimicrobial activity of outer membrane vesicles (OMVs). The graph shows *Dickeya dadantii* cells cocultured with phosphate buffered saline (PBS) as a negative control and its growth inhibition in the presence of *Pectobacterium brasiliense* 1692 OMVs and *Pbr*1692 cells. The results show the means of two independent experiments. Error bars represent the standard deviations from the data. Differences

between the effect of the PBS-negative control with *Pbr1692* cells and OMVs on *D. dadantii* was determined to be statistically significant with $p < 0.01$ in the analysis of variance.

Figure S2.1: Size distribution and concentration of nanoparticles in cell-free supernatants of exponential and stationary phase *Pectobacterium brasiliense* 1692 cultures.

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attach to the plant membrane to transfer AvrL, enabling AvrL entry into the plant cell. Once inside the cell, AvrL interacts with a number of host proteins, including transcription factors (TF) expressed in response to the *Pectobacterium brasiliense* 1692 infection. These interactions might either target the host proteins for degradation via the ubiquitin proteasome system (UPS) or inactivate them.

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CHAPTER ONE – Bacterial secretion system functions: evidence of interactions and downstream implications

Abstract

Unprecedented insights into the biology and functions of bacteria have been and continue to be gained through studying bacterial secretion systems in isolation. This method, however, results in our understanding of the systems being primarily based on the idea that they operate independently, ignoring the subtleties of downstream interconnections. Gram-negative bacteria are naturally able to adapt to and navigate their frequently varied and dynamic surroundings, mostly because of the covert connections between secretion systems. Therefore, to comprehend some of the linked downstream repercussions for organisms that follow this discourse, it is vital to have mechanistic insights into how the intersecretion system functions in bacterial rivalry, virulence, and survival, among other things. To that purpose, this paper discusses a few key instances of molecular antagonistic and interdependent relationships between bacterial secretion systems and their produced functional products.

Keywords: Secretion systems, intersecretion system crosstalk, nutrient acquisition, horizontal gene transfer, bacterial-host interaction.

Introduction

Systems of molecular secretion in bacteria promote pathogenicity and disease in diverse animal, human, and plant hosts. Depending on the lifestyle of the bacteria, secretion systems assume numerous key roles commonly entailing the transport of small molecules, nucleic acids, and proteins (Green & Mecsas, 2016). A suit of these secretory pathways dictates many aspects of bacterial biology that often maximise the success of bacteria and how pathogens impose adverse consequences on public, livestock, and plant health. For these reasons, knowledge behind the molecular mechanisms of these pathways has great implications for effective antivirulence drug discovery and subsequent management of bacterial infections.

Secretion systems are important for the biology of bacteria as transporters of proteins from the cytoplasm to the outer membrane and transporters of proteins from a donor cell to the environment or a recipient cell (Green & Mecsas, 2016). The latter type of transporters, which have evolved over time as nanomachines facilitating competition for resources and space, is the subject of a large number of excellent reviews that address, at large, how they facilitate bacterial competition and interaction with the environment, other bacteria, and hosts (Costa *et al.*, 2015, Green & Mecsas, 2016, Lynch & Alegado, 2017).

Secretion systems have mostly been analysed in Gram-negative bacteria (GNB) and, to some extent, Gram-positive bacteria (GPB) (Bernal *et al.*, 2018, Bowman & Palmer, 2021, Bunduc *et al.*, 2021, Rivera-Calzada *et al.*, 2021, Filloux, 2022). They are often designated as TXSSs, where X stands for any number from 1-11, including outer membrane vesicles (OMVs) (Figure 1.1), sometimes called a type zero secretion system (T0SS) (Chang *et al.*, 2014, Costa *et al.*, 2015, Guerrero-Mandujano *et al.*, 2017, Bunduc *et al.*, 2021). A major unifying thread among pathogenic bacterial secretory pathways, not related to type, is how they primarily assemble into channels for the transport of proteins including different virulence factors. Upon release, these proteins travel into and through biological barriers to interact with host components and specific immune factors, and subsequently promoting the reprogramming of several important cellular processes, ultimately leading to disease.

In light of recent advances in the understanding of bacterial secretion systems, the functional cooperation (i.e., several specialised secretion systems with a common

goal) among them or their products is an interesting research area as it contributes to bacterial survival, rivalry, and virulence. However, the field of bacterial interaction barely comprehend this notion, given the lack of knowledge on the elaborate bacterial responses through multiple secretion pathways. As a result, the prevailing mind-set on multisecretion system-mediated bacterial response may currently be slanted among the research community. Secretion systems are well organised along a bacterial cell where they can operate either independently or interdependently of each other. Additionally, and as we later discuss, these secretion systems can also communicate remotely via the release of their functional molecules (Figure 1.1).

Given that other reviews (e.g., Christie *et al.*, 2014, Nivaskumar & Francetic, 2014, Thomas *et al.*, 2014, Cianfanelli *et al.*, 2016, Deng *et al.*, 2017, Grohmann *et al.*, 2018, Van Ulsen *et al.*, 2018, Bhoite *et al.*, 2019, Palmer *et al.*, 2021) have already covered the fundamental functions of secretion systems, we focused heavily in our review on the interconnections between bacterial secretion systems and how they impact bacterial biology. In order to further emphasise the importance of investigating bacterial secretion system interaction, we additionally offered a few previously underestimated functions that are mostly apparent during the interaction of the secretion systems, and provided their possible downstream implications.

The general functions and biology of bacterial secretion systems

TXSSs

With the exception of the T1SS, whose function is restricted to the dispersal of substrates into the external environment, the T3SS, T4SS, and T6SS of GNB are widespread double membrane spanning one step translocators of proteins into the environment or target cells without periplasmic passage (Figure 1.1). The latter are contact-dependent injectosomes of GNB (Galan & Waksman, 2018). Recently, it has been shown that the mechanism for T1SS in some cases is a two-step process, and through bacteriocin-like protein surface aggregates, T1SS can facilitate antibacterial competition in a contact dependent manner (García-Bayona *et al.*, 2017, Spitz *et al.*, 2019, Smith *et al.*, 2020). This system typically secretes diffusible bacteriocins, adhesins, and proteins required during nutrient acquisition, such as iron scavenger proteins, lipases, proteases, and pore-forming toxins important for survival and pathogenesis (Kanonenberg *et al.*, 2013, Kanonenberg *et al.*, 2018). The T3SS and

T6SS secrete effectors primarily involved in host immune subversion or manipulation and bacterial competition, respectively (Buttner & He, 2009, Russell *et al.*, 2011, Durand *et al.*, 2014, Galán *et al.*, 2014, Wagner *et al.*, 2018, Smith *et al.*, 2020). Both these systems are also involved in other roles such as symbiotic interactions including mutualism, commensalism, and pathogenesis (Egan *et al.*, 2014, Hachani *et al.*, 2016, Hu *et al.*, 2017, Bernal *et al.*, 2018, Speare *et al.*, 2018, Lucke *et al.*, 2020, Borrero de Acuña & Bernal, 2021). As recently hypothesised, pathogenic bacteria may also use the T3SS to indirectly target microbiota populations (Liu *et al.*, 2022). On the other hand, additional roles of the T6SS have come to light in recent years, including anti-fungal activities and extracellular metal uptake (Russell *et al.*, 2014, Lien & Lai, 2017, Coulthurst, 2019, Teschler *et al.*, 2022). The T6SS has recently been demonstrated to affect the competition between predator and prey cells inside the biofilm (Teschler *et al.*, 2022) and to transport a DNase that kills fungus by damaging their DNA via a Mg^{2+} -dependent mechanism (Luo *et al.*, 2023). Unlike other TXSSs, excluding membrane vesicles (T0SS), the T4SS appears to have a unique function of delivering DNA and protein-DNA complexes, suggesting it is a tool for horizontal gene transfer (HGT) and, importantly, for spreading resistance genes among bacterial communities (Juhas *et al.*, 2008, Huddleston, 2014). However, the T4SS also shares some functions with other TXSSs, including bacterial killing and delivering effectors into host cells, functions previously thought to be unique to the T6SS and T3SS, respectively (Bayer-Santos *et al.*, 2019, Souza *et al.*, 2019). Recent studies have revealed insights into the structure, function, regulation, and role of the T4SS in bacterial pathogenesis and host-pathogen interactions (Sheedlo *et al.*, 2022, Purtschert-Montenegro *et al.*, 2022). The T2SS, T5SS, T8SS, T9SS, and T11SS are the GNB two-step transporters, among which, only the T2SS and T9SS act as transenvelope machines, spanning both the inner and outer membrane of the bacterial cell envelope (Sato *et al.*, 2010, Green & Mecsas, 2016, Veith *et al.*, 2017). Components of the T10SS that facilitate the release of substrates from the periplasm to the extracellular space have not been described (Hamilton *et al.*, 2014). The T2SS substrate repertoire continues to expand and includes typical substrates for bacterial adaptation and nutrient acquisition such as hydrolytic enzymes and toxins (Costa *et al.*, 2015, Cianciotto & White, 2017). Most substrates of the T5SS, and similarly of the T9SS, remain attached to the outer membrane and a few are released into the extracellular environment (Filloux, 2022).

Membrane vesicles

Bacterial membrane vesicles are arguably some of the most impressive features of microbes that have recently intrigued many scientists in the field of bacterial interactions. As vesicles exhibit some differences in transmission of molecular cargo compared to TXSSs, a brief discussion is warranted. Traditionally regarded as an inert membrane anomaly, membrane vesicles are frequently released by species in all three branches of life as subcellular lipid-bilayers (20-500 μm in diameter) (Gill *et al.*, 2019). According to a decades' worth of research, membrane vesicles play a significant role in cell-cell and interorganismal communication due to their ability to internalise cellular contents (e.g., signalling molecules, toxins, proteins, metabolites, and nucleic acids) in their lumen. In this way, enclosed luminal contents maintain their potency because they are protected from extracellular degradative enzymes, making them highly concentrated and easy to deliver over long distances (Bonnington & Kuehn, 2014). Upon release, membrane vesicles can either persist in the extracellular environment for long periods of time or fuse with prokaryotic or eukaryotic cells, for instance, via raft-dependent endocytosis, eliminating the need for microbes to have close contact with the host in order to rapidly transfer their cargo (Bomberger *et al.*, 2009).

Bacterial membrane vesicles include outer membrane vesicles (OMVs), explosive outer membrane vesicles (EOMVs), cytoplasmic membrane vesicles (CMVs), and outer-inner membrane vesicles (OIMVs), mainly containing outer membrane (OM), cytoplasmic membrane, and outer and inner membrane (IM) components, respectively (Toyofuku *et al.*, 2019). CMV, EOMV, and OIMV biogenesis happens through several proposed routes including explosive cell lysis via cryptic prophage endolysin activity (Toyofuku *et al.*, 2019). OMV production entails unbalanced biosynthesis of cell wall components or the intercalation of hydrophobic molecules with the outer leaflet of the OM, leading to membrane curvature and eventual outgrowth of the OM (Schertzer & Whiteley, 2012, Toyofuku *et al.*, 2019, McMillan & Kuehn, 2021). For the purpose of this review, here on, all the vesicle types will be referred to as OMVs, for which formation is accompanied by the internalization of cellular contents delimited from extracellular enzymes degradation enclosed in the vesicle membrane (Bonnington & Kuehn, 2014).

The role of secretion systems: A case in *Pectobacterium* spp. survival and pathogenicity

Several phytopathogens, including Soft Rot *Pectobacteriaceae* threaten crop and ornamental production globally. *Dickeya* and *Pectobacterium* are two genera that colonise plants, insects, water, and soils leading to necrotic rot, wilt, and blackleg diseases on plants. Some *Pectobacterium* spp. represent highly virulent phytopathogens within SRP. These bacteria employ different mechanisms dedicated to adapting to changing environments and a wide range of hosts (Oulghazi *et al.*, 2021). These include virulence determinants such as quorum sensing, secretion systems, PCWDEs, proteases, hemagglutinins, adhesins, iron acquisition systems, secondary metabolites, toxins, chemotaxis, flagella, and motility (Glasner *et al.*, 2008, Effantin *et al.*, 2011, Charkowski *et al.*, 2012, Davidsson *et al.*, 2013, Nykyri *et al.*, 2013, Moleleki *et al.*, 2017). *P. brasiliense* causes pre-emergence seed rot, aerial stem rot, blackleg in the field and post-harvest storage soft rot of potato tubers of its primary host potato (van der Merwe *et al.*, 2010, Onkendi & Moleleki, 2014, de Werra *et al.*, 2015, Fujimoto *et al.*, 2017, Voronina *et al.*, 2019). The virulence of soft rot *Pectobacterium* spp. is exacerbated by ineffective treatment methods, a limitation that largely exists because of the paucity of knowledge on the biology particularly available for this pathogen (Czajkowski *et al.*, 2011, Oulghazi *et al.*, 2021). Seven secretion systems including OMVs, with some unique virulence factors they export, have been recognised as an important component of some *Pectobacterium* spp. pathogenicity and aggressiveness on a broad host range (Glasner *et al.*, 2008, Charkowski *et al.*, 2012, Li *et al.*, 2018, Bellieny-Rabelo *et al.*, 2019, Shyntum *et al.*, 2019, Piotrowska *et al.*, 2020).

Outer membrane vesicles of a few *Pectobacterium* spp. have been characterised. The vesicles transport diverse cargo in their lumen, which is essential for virulence and survival (Piotrowska *et al.*, 2020, Jonca *et al.*, 2021). Most T1SSs of SRP secrete metalloproteases involved in protein and host cell wall degradation. *P. atrosepticum* T1SS also secretes protein multi-repeat adhesin (Pérez-Mendoza *et al.*, 2011). The T2SS is the primary virulence determinant of SRP, secreting the PCWDEs responsible for the maceration of plant tissue (Davidsson *et al.*, 2013, Chang *et al.*, 2014). Among the secretion systems of SRP, T3SS is not highly conserved and does not appear to have a central role in the pathogenesis of some *Pectobacterium* spp. (Davidsson *et*

et al., 2013, Gorshkov *et al.*, 2018). In addition, the substrates of the T4SS are unknown; thus, its function is unclear. The T5SS is involved in contact dependent inhibition (Glasner *et al.*, 2008, Poole *et al.*, 2011, Charkowski *et al.*, 2012). The T6SS is implicated in bacterial-bacterial, bacterial-environment, and bacterial-host interactions (Coulthurst, 2019). *P. brasiliense* 1692 T6SS is involved in bacterial competition (Shyntum *et al.*, 2019). However, the T6SS does not appear to mediate any observable competition within potato leaves (Miguel, 2020). Furthermore, several putative antibacterial effectors ectopically expressed in *Escherichia coli* suggested that the T6SS's primary function within potato leaves is not bacterial competition (Miguel, 2020). Unlike in animal pathogenic GNB, seldom are phytopathogenic T6SSs associated directly with pathogenesis. In fact, there are very few experimentally validated type 6 secreted effectors in general. In molecular plant-pathogen interactions, the virulence role of T6SS remains a hypothesis supported by early suggestions such as those relating to the T6SS secreted Hcp protein of *Agrobacterium tumefaciens* DC58, earlier proposed to promote potato tumorigenesis (Wu *et al.*, 2008). Since then, several studies have reported virulence phenotypes in T6SS mutant strains and upregulation of the T6SS cluster genes in wild type strains during *in planta* host infection (eg., Liu *et al.*, 2008, Mattinen *et al.*, 2008, Syntum *et al.*, 2015, Bellieny-Rabelo *et al.*, 2019). Expression profiling data from *Pbr1692* indicated upregulation of T6SS at different time points during potato tuber infection, which in turn suggests a possible role in pathogenesis (Bellieny-Rabelo *et al.*, 2019).

Intersecretion system-mediated response in bacteria

In this section, we explore some of the molecular mechanisms underlying the interconnectivity of the different secretion systems, and we show how this leads to several important functions, such as collaborative assault, setting-up intimate bacteria-host and bacteria-bacteria contact, exploitative competition, and horizontal gene transfer (HGT) in plant and animal bacterial pathogens as well as non-pathogenic bacteria. In Figure 1.1 and Table 1.1, the interaction-mediated functions have been summarised, which, for the purpose of discussions in this section, are described in terms of substrate and system level interaction.

Host cell attachment and damage coupled to effector translocation

Contact with the host surface is perhaps one of the most important features of bacteria in initiating an infection, and it can trigger events in the host cell that promote pathogen's internalization and continuation of its life cycle (Kenny *et al.*, 1997, Mao *et al.*, 2017). This is typically carried out by several T5SS classes, namely classical autotransporters (T5aSS), two-partner secretion (T5bSS), trimeric autotransporter adhesins (T5cSS), T5dSS, inverse autotransporters (T5eSS), and T5fSS (Meuskens *et al.*, 2019). Several of these T5SS classes reportedly function together with the T3SS and T4SS in establishing contact between the pathogen and the cognate adhesin receptor found on the host surface, and in some cases even driving cytoskeletal rearrangements and effector-triggered host cell invasion (Mix *et al.*, 2021). A classic example is the T3SS-dependent translocation of Tir (Translocated Intimin Receptor), a protein typically produced by enteropathogenic *Escherichia coli* (EPEC) bacteria; EPEC causes diarrhoea, the primary cause of morbidity and mortality among children in developing countries (Kotloff *et al.*, 2013). Tir plays a crucial role in the pathogenesis of EPEC infections and subsequent development of paediatric diarrhoea by promoting attachment of pathogenic *E. coli* cells to host intestinal cells and inducing changes in host cell behaviour. Following attachment, the T3SS of EPEC delivers a suit of effector proteins into the host cell, including Tir (Kenny *et al.*, 1997). As illustrated in Figure 1.1, Tir becomes localised to the plasma membrane via the host Golgi apparatus (Mao *et al.*, 2017), then acts as a receptor for a T5eSS substrate of the bacterial protein, Intimin (Kenny *et al.*, 1997). The established Tir–intimin linkage allows EPEC to form a tight adherence to the host cell, known as attaching and effacing (A/E) lesions, which emerge as a result of the effacing of the small intestine lining, a hallmark of EPEC infections.

The *Yersinia* adhesin A (YadA) from *Yersinia enterocolitica* and BadA (*Bartonella* adhesin A) from *Bartonella henselae* both fall under the T5eSS class. They engage in an intersecretion system-mediated translocation of effector molecules via the T3SS and T4SS (Keller *et al.*, 2015, Mix *et al.*, 2021). In particular, system interaction between BadA (T5cSS) and a T4SS core VirB/D-like subcomplex in *B. henselae* reduces T4SS mediated pathogen virulence (Lu *et al.*, 2013), meaning the interaction is antagonistic unlike in EPEC (Kenny *et al.*, 1997). Thus, most human *B. henselae* isolates were observed to have lost either BadA or VirB/D4 T4SS (Lu *et al.*, 2013).

VirB/D4 denotes a structure of the T4SS that entails VirB1-11 proteins that assemble to form a secretion machinery and a pilus (T4P), and the VirD4 protein that is liable for substrate recruitment to the T4SS for secretion through the translocation channel (Redzej *et al.*, 2017). The VirB/D4 T4SS function is thus interrupted by BadA which, through its effective length, enforces a physical distance (space) between the pathogen's outer membrane (OM) and host cell membrane (Lu *et al.*, 2013). Subsequently, this negatively impacts the VirB/D4 T4SS effector translocation efficiency and ultimately impedes pathogen virulence. Considerations were made for possible shortfalls of the study resulting in the observed antagonism. It is possible that, in isolates where BadA and VirB/D4 co-exist to ensure persistence and effective infection, the strains likely express *BadA* or *VirB/D4* genes at different stages of host infection to, in part, minimise a speculated risk of cell wall instability due to protein overload in the OM. Also, given that most strains with either one of these virulence factors are observed, it is highly likely that they are antagonistic in these strains or the VirB/D4 is barely useful to retain in the presence of BadA. Such potential interactions have been missed in the literature as a result of isolated analysis of key virulence factors or the assumption that all secretion systems follow the same virulence factor-interaction strategies (Lu *et al.*, 2013). Observations relating to similar bacteria-host cell contact mechanisms that are mediated by secretion system interplay have been made for *Helicobacter pylori*, *Pseudomonas aeruginosa*, and *Salmonella enterica* (Sundin *et al.*, 2002, Miyake *et al.*, 2005, Backert & Selbach, 2008, Gerlach *et al.*, 2008, Backert *et al.*, 2011, Ishijima *et al.*, 2011, Mix *et al.*, 2021).

The T2SS and T3SS are identified as some of the key virulent determinants in bacteria and have been reported multiple times to promote host cell damage and virulence, respectively, in gram-negative pathogens of plants and animals. It is not surprising that these two systems act in concert to achieve a bunch of objectives in bacteria. Specifically in plant infections, the T2SS of bacterial pathogens primarily export an arsenal of plant cell wall degrading enzymes (PCWDEs), such as pectate lyase, pectin lyase, polygalacturonase, cellulase, and protease (Cianciotto & White, 2017). In *Dickeya dadantii*, a broad-host-range enterobacterium belonging to plant pathogenic soft rot pathogens, there is an interplay between the T1SS and T2SS at the substrate level. During this interplay, T1SS extracellular protease PrtA post translationally modifies the T2SS-dependent pectate lyase (Pell-2) by cleaving its *N*-terminal amino

acids (Shevchik *et al.*, 1998). The resultant protein is a small, slightly more basic, and more efficient necrosis-inducing protein called Pell-3 (Shevchik *et al.*, 1998). PCWDEs such as Pell are delivered to the host cell surface whereby they act to reduce cell wall integrity, thereby establishing a pathogen's nutrient supply line that is based on the cell wall components of the host plant. For this reason, it is conceivable that a portion of the cell wall-derived components will be utilised in the assembly of bacterial weapons including secretory channels of virulence factors, including the T3SS (Buttner & He, 2009). Here, we also demonstrate an interaction between this system and the T2SS in *Xanthomonas campestris* pv. *vesicatoria*, the causative agent of bacterial spot of pepper and tomato. The T2SS activity is thought to disrupt the plant cell wall by releasing hydrolytic enzymes that allow T3SS-dependent effector protein translocation (Szczesny *et al.*, 2010). The cell wall-degrading activity in *X. campestris* pv. *vesicatoria* is specifically carried out by the Xps-T2SS, one of the two T2SSs spanning the envelope of this pathogen (Szczesny *et al.*, 2010). Szczesny and co-workers (2010) hypothesised that degradation of the plant cell wall helps in nutrient acquisition and that T2SS PCWDEs might facilitate the assembly of extracellular components of T3SS pili for effector injection into the host cell. The study highlighted that mutation of the Xps system reduced translocation of T3 effectors but did not markedly affect the T3SS or the synthesis of its components (Szczesny *et al.*, 2010). Traces of plant cell wall could be observed in some studies after it has been degraded. It is possible that PCWDEs weaken the plant cell wall for effective T3SS effector translocation. Further investigation is required to validate these postulated synergistic functions. Pili are often used for host penetration by bacteria since they can span the thick plant cell wall to translocate effectors into the target plant. Similar structures that are conserved in bacterial pathogens suggest that the T2SS shares composition and structural features with the T4SS pili (T4P) (Korotkov *et al.*, 2012), important in bacterial adherence to host cells and other surfaces (Hospenthal *et al.*, 2017). This enforces the notion of a common origin and potentiates pilus-mediated secretion, which is also involved in secretion system interaction (discussed later in this section). The interaction of the T2SS and T3SS is also tightly linked to the regulation of host defences, as well as the expression of genes and substrates of both systems, which is regulated by the HrpG/HrpX regulon (Jha *et al.*, 2007, Szczesny *et al.*, 2010, Guo *et al.*, 2011, Alvarez-Martinez *et al.*, 2021).

A previously unrecognised iron acquisition and horizontal gene transfer mechanism

Direct physical contact of one bacterial cell with cells in the vicinity is an essential component of bacterial survival within a community. The T6SS is a bacteriophage-like machinery that is usually deployed during bacteria-bacteria contact where it delivers toxic effectors directly into neighbouring cells of competitor bacteria. All things considered, the T6SS is mostly regarded as a contact-dependent system, however, as we will show later, it can also engage in contactless exercises through interaction of its substrates with OMVs. Currently, there is no general mechanisms defining exactly how bacteria release, recognise, and recruit OMVs in an intra and inter-specific manner. However, two recent studies have revealed some key molecular mechanisms that might be involved in some of these processes in *P. aeruginosa* and *Cupriavidus necator*, entailing T6SS-mediated recruitment of OMVs during species communication (Lin *et al.*, 2017, Li *et al.*, 2021). There are some aspects of this recruitment mechanism that are shared by both bacterial pathogens, and may also be found in other species. To begin, the T6SS effectors are employed as an OMV recruitment tool, and when OMVs are brought into play, a non-contact apparatus in the form of T6SS-OMV emerges (Lin *et al.*, 2017, Li *et al.*, 2021). This makes reasonable sense as research shows that bacterial species can respond to environmental stimuli thanks to the release of OMVs (Costa *et al.*, 2015). Iron is an important metal for bacterial survival and virulence. *P. aeruginosa* H3-T6SS promoters in PA Δ 3Fe (an iron acquisition mutant strain defective in the pyoverdin biosynthetic pathway (Δ pvdA), the pyochelin synthetase (Δ pchE) and the ferrous iron transport (Δ feoB)) are induced in iron-deficiency conditions (Lin *et al.*, 2017). Under iron depleted environments, *P. aeruginosa* is able to scavenge iron from the extracellular 2-heptyl-3-hydroxy-4-quinolone (PQS), mostly found in OMVs. First, TseF, a H3-T6SS secreted effector, directly interacts with the iron acquisition receptor, FptA, then PQS, whose affinity with TseF increased in the presence of iron. Ultimately, TseF indirectly facilitates iron acquisition by delivering the OMV associated Fe³⁺-PQS complex iron to *P. aeruginosa* cells in a PQS dependent manner. More so, TseF bridging of the interaction between *P. aeruginosa* OMV-bound iron binding molecule PQS and *P. aeruginosa* cells by directly binding FptA ((Fe(III)-pyochelin receptor)) and porin OprF, consequently directs iron to the cells for uptake via an unknown mechanism. As expected, the growth of TseF and H3-T6SS mutants in iron depleted or Fe³⁺-PQS supplemented media,

even in the presence of functional iron receptors, is severely affected in the absence of the recruiting effector, TseF (Lin *et al.*, 2017).

The delivery of effectors or ions into target cells, even at a distant, from the site of colonization presents a unique population feeding advantage over traditional secretion systems that are often tightly fixed to the membrane and peptidoglycan layer of the cells. As noted previously, the T6SS is primarily depended on contact to induce an effect on the recipient cells. Therefore, by recruiting OMVs through the incorporation of effectors into OMVs, the T6SS may perform its functions without proximity restrictions. Second, effectors that associated with OMVs for function or transport might also direct OMVs to the bacterial cell surface where they interact with specific OM receptors involved in iron uptake (Lin *et al.*, 2017, Li *et al.*, 2021). Third, under iron-diluted conditions, activation of surface-associated receptors facilitates the delivery of iron to the cytosol, and once replete, dissolved iron can promote the competitive ability of *P. aeruginosa* and *C. necator* in their respective environments (Lin *et al.*, 2017, Li *et al.*, 2021). When all of these factors are considered, a novel function of the T6SS, seen to promote the efficient utilization of iron when its sources are running low seems to be an intricate one.

We further consider the unique feature at play during the interaction of the T6SS with OMVs that is required for the bacteria to thrive in an iron-diluted environment. First, in *P. aeruginosa*, some aspect of iron uptake is facilitated by a newly described effector, called PA2374 or TseF (Figure 1.1), leading to the delivery of iron into *P. aeruginosa* cells (Lin *et al.*, 2017). In *C. necator*, a ligand (lipopolysaccharide)-receptor (CubA and CstR) interaction mediated inter-species OMV recruitment mechanism is observed. The LPS-binding effector TeoL (T6SS effector for recruitment of OMVs via lipopolysaccharide (LPS)) especially recognises and binds the LPS on OMVs from different species (e.g., *C. necator* and distantly related *P. aeruginosa* PAO1 and *Yersinia pseudotuberculosis* YPIII) containing the iron-chelating molecule PQS. Next, CubA (cupriabactin siderophore receptor) and CstR (catecholate siderophore receptor), both of which part of the bacterial OM and essential for the ability of bacteria to grow and survive in iron-poor environments, are activated, eventually leading to ligand-receptor interaction-based OMV recruitment (Li *et al.*, 2021). The O-antigen component of OMVs, which is a carbohydrate structural region of the LPS, and the

compositional differences in LPS between bacterial cells and OMVs (e.g., partial loss of the LPS in OMVs), contribute to TeoL's preferential binding to bacterial OMVs rather than to bacterial cells (Li *et al.*, 2021). In addition to iron uptake, the OMVs recruited by TeoL were observed to be important for exploitative competition, resistance to oxidative stress, and HGT in recipient cells—key in their survival and persistence. Given the fact that PQS and LPS are involved in OMV production during a process entailing PQS-mediated anionic repulsions between the LPS molecules (Mashburn-Warren *et al.*, 2008), it would be interesting to examine the possibility of effector-induced vesiculation in bacteria as a way to increase nutrient sources that are depleted. This brings up the issue of whether OMVs could be utilised as a wellspring of nutrients or factors significant for nutrient acquisition, and whether TXSS effector-mediated recruitment of OMVs is a general survival mechanism used by bacteria. Nonetheless, the discoveries of these studies certainly illuminate how we might interpret the role of bacteria in recognizing and recruiting OMVs for various survival and host invasion strategies.

T6SS-mediated recruitment of OMVs also enables bacteria to participate in HGT. OMVs can drive HGT and bacterial resistance to stress (Klieve *et al.*, 2005, Fulsundar *et al.*, 2014, Mozaheb & Mingeot-Leclercq, 2020), but to our knowledge, they have seldom been associated with other TXSSs in accomplishing HGT. Recently, it was established that the T6SS can promote HGT by enabling acquisition of DNA from OMVs purified from bacterial cultures containing plasmid DNA (Li *et al.*, 2021). As previously discussed, pilus-mediated interaction in terms of intersecretion system crosstalk plays a key role in bacterial contact with either other bacteria or the host. A key example of this is a pilus-mediated interaction in terms of HGT that involves a crosstalk between the T4SS and T6SS (Figure 1.1) (Ho *et al.*, 2013). This is consistent with HGT encompassing T4SS-mediated cell-cell contact through conjugative DNA transfer in bacteria (Hospenthal *et al.*, 2017). The T4SS-mediated HGT was found to activate T6SS-mediated killing of adjacent donor cells carrying parasitic foreign DNA, and is considered an “innate immune system” that recognises transfer-associated patterns instead of molecular patterns of infectious elements (Ho *et al.*, 2013). When contact-dependent killing via intersecretion system crosstalk is involved, this suggests that HGT is undeniably more complex than previously thought.

Intersecretion system-mediated microbe-microbe contact

The interaction between the T6SS and T4SS has recently been observed to play a role during contact between non-pathogenic *Neisseria cinerea* and other human pathogens (Custodio *et al.*, 2021). As mentioned previously, T4Ps form part of the VirB/D4 T4SS design, and are found on bacteria's surfaces, aiding in bringing bacteria into close physical association with host cells and other bacteria (Craig *et al.*, 2019). In competition assays involving human commensal and pathogenic strains of *Neisseria*, *N. cinerea* was instrumental in killing pathogens, *N. meningitidis* and *N. gonorrhoeae*, in a T6SS dependent manner (Custodio *et al.*, 2021). Prey strains of *Neisseria* lacking a T4P, in particular, were able to escape the T6SS-mediated killing by segregating to the edge of the colony seeded on agar medium. However, prey strains that expressed a pilus were outcompeted by the killer strain due to cellular interaction between themselves and *N. cinerea*, which was mediated by a T4P. This implies that T4P promotes the activity of contact dependent TXSSs by bringing prey closer to the attacker. It is not uncommon for bacteria to influence the outcome of an infection by directly killing other bacteria through the antibacterial action of the T6SS. This has been associated with changes in microbial communities and a range of ecological consequences (Gallegos-Monterrosa & Coulthurst, 2021). Therefore, intersecretion system as a function of pilus-mediated interaction may greatly contribute to microbial community structures and composition, which is reminiscent with the role of the T6SS (Gallegos-Monterrosa & Coulthurst, 2021).

Shared TXSS substrates highlight a conceivable interplay

Although there are a number of studies which do not directly dissect bacterial secretion system dialogue, they give significant insights to their possible interaction and set the stage for future research. For example, the *P. aeruginosa* T3SS and T6SS may work together to regulate transcription factors that activate unique transcriptome changes during early airway epithelial cell infection (Sen-Kilic *et al.*, 2022). With respect to the T3SS and T4SS, we speculate functional interplay could be a possible outcome since the effectors of these secretion systems are often observed to be remarkably alike in structure and function (An *et al.*, 2017). This means that the functional secretion system might act in place of the mutant system and secrete its substrates. Alternatively, the functional secretion system substrates might substitute the mutated

systems' substrates. In addition, several studies on bacterial secretion suggests that many substrates are shared between OMVs and TXSSs. For instance, OMVs are known to export many proteins which play a role in bacterial virulence and communication, and many of these are shared with TXSSs (Bonnington & Kuehn, 2014). In plant-associated bacteria, a large number of studies reported diverse proteins in OMVs that are biologically important (Janda *et al.*, 2022). An overlap in substrates between the T2SS and OMVs was observed, whereby *X. campestris* pv. *vesicatoria* strains lacking a functional T2SS independently secreted several substrates of the T2SS system, including extracellular protein cargoes such as lipases, proteases, and cell wall-modifying enzymes via OMVs (Solé *et al.*, 2015). Likewise, OMVs isolated from phytopathogens overlap in extracellular protein cargo with the T2SS and T3SS (Chowdhury & Jagannadham, 2013, Nascimento *et al.*, 2016). Both T2SS secreted hydrolytic enzymes and the T3SS effectors whose translocation into host cells is potentially facilitated by them were found in OMVs of *P. syringae* pv. tomato T1 (Chowdhury & Jagannadham, 2013). In addition, OMV-mediated transport of biologically active T2SS dependent PCWDEs has been reported in *Pectobacterium* spp. *P. odoriferum*, *P. versatile* and *P. zantedeschiae* and *X. fastidiosa* vesicles (Feitosa-Junior *et al.*, 2019, Jonca *et al.*, 2021). Taken together, these analyses suggest that OMVs could serve as an alternative secretory pathway for other TXSSs in bacteria or act in coordination with them.

Implications for the inter-secretion system dialogue

Interaction between secretion systems could be a common occurrence in prokaryotic organisms. How it impacts bacterial function and interactions is not yet clear, but it perhaps represents an ingenious mechanism that ensures bacteria use adequate tools at the right time to enhance their fitness potential. In this section we consider some of these specific aspects and their potential consequences.

Outer membrane vesicles shared as “public goods”

Although OMVs were originally thought to be merely membrane artefacts with no clear cellular importance, in recent decades, an enormous number of investigations gave an account of their functions including in nutrient acquisition and exploitative competition. For example, OMVs from *Mycobacterium tuberculosis* can carry high amounts of an iron chelating molecule, mycobactin, which forms iron-scavenging OMVs

(Prados-Rosales *et al.*, 2014). Once released into the environment, these myobactin-OMVs can be shared *bona publica* as they will be relatively easy to access by neighbour bacteria as a community resource, thereby contribute to the social life of that community (West *et al.*, 2007). In essence, OMVs can serve an ecologically significant role for the successful coexistence of different bacterial species in the same habitat or within a biofilm—structured communities of microbial aggregates enclosed in a self-produced polymeric matrix and attached to biological and non-biological surfaces. Here, we consider these ideas in the context of OMV interaction, which so far have been reported with the T6SS and T9SS. As previously noted, *P. aeruginosa* secretes OMVs carrying on their surface the PQS molecule, which, like myobactin, also strongly binds iron. These PQS-containing OMVs can be recruited by the T6SS effector TseF (Lin *et al.*, 2017), and possibly by other members in the bacterial community that express the T6SS effector. Although further research is needed to determine the entire range of bacteria that secrete this effector, it is possible that other bacteria may also produce the effector. Additional proof of OMVs as potential public goods relates to features of the bacterial OM. The LPS, which is one of the major constituents of bacterial cells and OMVs, is important for the cell envelope of GNB, serving principally as a structural component of OM and released OMVs. Likewise, there is solid support of OMV-mediated conveyance of the LPS among bacterial species which can also trigger important host cellular processes (Vassallo *et al.*, 2015, Vanaja *et al.*, 2016, Glew *et al.*, 2021). This solidly addresses the ability of bacterial cells to disperse significant surface-confined particles even to locales where OMV donor cells themselves cannot reach. Along these lines, the T6SS-mediated recruitment of OMVs may hypothetically be an element of bacteria traded among neighbours and as a public good. Considering that the T6SSs are present in a wide spectrum of gram-negatives (>25% of Proteobacteria) (Bingle *et al.*, 2008), the T6SS-mediated recruitment of OMVs could therefore be an alternative mechanism of utilizing scarce iron from its dilute sources exploited by many bacteria. In addition, iron acquisition in this manner also brings about other benefits, as we have seen that the capacity of the T6SS effectors to recruit OMVs also prompts other important functions, including HGT and stress tolerance, that are critical for bacterial rivalry and survival while sharing the same habitat (Schwechheimer & Kuehn, 2015, Lin *et al.*, 2017, Li *et al.*, 2021). In a similar scenario, the T9SS, a translocon in a few *Bacteroidetes* spp. including *Porphyromonas gingivalis*, interacts with OMVs via a battery of virulence

effectors (Figure 1.1) (Veith *et al.*, 2021). These include extracellular cysteine proteases, representing hallmark virulence factors of this pathogen, commonly called gingipains (RgpA, RgpB, and Kgp) (Veith *et al.*, 2021). These virulence factors carry a conserved C-terminal domain (CTD) that is used as an extracellular OM translocation signal by the T9SS (Glew *et al.*, 2012). Following translocation, the CTD is cleaved off by a bifunctional C-terminal signal peptidase and sortase enzyme, PorU. The sortase is also released via the T9SS but, unlike gingipains, it retains the CTD signal (Glew *et al.*, 2012, Gorasia *et al.*, 2015, Veith *et al.*, 2020), after which its replacement, as well as that of gingapins, with a unique anionic lipopolysaccharide (A-LPS) moiety of *P. gingivalis* OM effectively anchors the released proteins to the OM, forming a virulence coat on the cells and OMVs (Veith *et al.*, 2020, Madej *et al.*, 2021, Veith *et al.*, 2021). Because the LPS can be exchanged between bacterial strains, the OMVs coated with virulence factors (virulence factor-coated) may be released into the extracellular space, where they will most likely be shared as public goods (Vassallo *et al.*, 2015, Glew *et al.*, 2021). In this way, the virulence coat can be spread by released OMVs, and in some cases even to other non-pathogenic *P. gingivalis* strains within the pathogen population. Noteworthy, bacteria can trade certain traits through the use of OMVs (Vassallo *et al.*, 2015). Such a vesicle-mediated exchange of cargo or traits is not confined to bacteria. In the yeast genus *Cryptococcus*, for instance, extracellular vesicles released by virulent strains can trigger a rapid intracellular proliferation of non-virulent yeast strains residing within the macrophages (Bielska *et al.*, 2018). This results in pathogenic ‘division of labour’, which occurs remotely where vesicles diffuse over large distances, in the process possibly transmitting virulence factors to these strains. Alternatively, it is possible that the *Cryptococcus* vesicles can modify the host environment to allow less pathogenic strains to thrive, which will also facilitate the pathogenic ‘division of labour’. The same ‘division of labour’ phenomenon may apply in *P. gingivalis* strains when the T9SS virulence coat is distributed from a few pathogenic members to otherwise latent and non-virulent strains.

Conjugation is a widespread channel of HGT, a consequence of which is rapid evolution and adaptation of bacterial strains through the spread of antimicrobial resistance genes (Virolle *et al.*, 2020). Plant-derived bioactive compounds identified to inhibit T4SS-mediated conjugal transfer of plasmids without perturbing GNB growth were reported not long ago (Oyedemi *et al.*, 2016). However, in addition to conjugation

and transformation by the T4SS, OMVs are also disseminators of antibiotic resistance genes in GNB, and cultures supplemented with purified vesicles are protected from antimicrobial compounds by several strategies such as drug binding (Manning & Kuehn, 2011, Rumbo *et al.*, 2011, Kulkarni *et al.*, 2015, Chatterjee *et al.*, 2017). In *Vibrio cholerae*, a protease essential in bacterial resistance to host antimicrobial peptides and conventionally secreted via the T2SS, is associated with OMVs (Rompikuntal *et al.*, 2015). Being evolutionarily conserved, constitutive, and primarily produced in response to stress, dissemination of resistance via OMVs may soon become notably problematic as resistance genes continue to be availed to complex microbial communities (Li *et al.*, 2016). We are likely to observe roles beyond OMV-T2SS, -T3SS, -T4SS, -T6SS, and -T9SS associations as vesicles are handy and convenient sources (i.e., public goods) of protected and concentrated bioactive compounds for mixed communities (Lynch & Alegado, 2017, Toyofuku *et al.*, 2017).

Differential secretion of substrates

The focus of profiling secretion systems includes proteomic research. To demonstrate secretomes that are representative of the function of the missing system, secretion system knockout mutants are often constructed. In proteomic investigations, it has been revealed that proteins are downregulated in TXSS mutant strains compared to wild type strains in the hunt for the TXSSs' major substrates. It is possible that the differentially secreted proteins are an indication of secondary protein secretion or of regulation by the disrupted system. The key to bacterial fitness and survival in the environment is synchrony. Bacteria intentionally stimulate protein secretion for this reason so they can react quickly to environmental changes (Leung *et al.*, 2011). In this way, regulatory networks, and secretion systems along with their crosstalk play a critical part in the communication, which is in turn critical for fitness in all forms of life (Yeo *et al.*, 2021). For instance, a recent study identified new *Dickeya dadantii* T2SS substrates displaying a band of low intensity and thickness for one protein, VirK, in the supernatant of the T2SS inactive strain as compared to the supernatant of the T2SS active strain or the complement (Condemine & Le Readout, 2022). VirK could have leaked, or in our judgment was a subject of secondary secretion by OMVs or another system thus the cause of its appearance in T2SS mutant secretomes. Similarly VirK and a cellulase were previously only discovered in the minimum media with extract of the T3SS active strain secretome of the phytopathogen *X. citri subsp. citri*. (Ferreira

et al., 2016). The study attributed the outcome to the dysfunctional T3SS system in the mutant strain or that the T3SS apparatus had pleiotropic effects on the expression and secretion of some proteins. However, since another study demonstrated in *Ralstonia solanacearum* that HrpG of the T3SS regulated VirK, it was concluded that the T3SS controls T2SS secretion (Ferreira *et al.*, 2016). A study in *Salmonella* identified the crosstalk between the T6SS encoding SPI-19 and the T3SS in avian infection, which is another recent discovery (Zhang *et al.*, 2022). The research revealed that T3SS regulators made up part of the T6SS island. Genes that make up the T6SS core components were not a part of the observed regulation (Zhang *et al.*, 2022). All things considered, regulatory or polar effects of some mutations may be at play. Nonetheless, it is crucial to keep in mind that secretion systems could be involved in co-secretion and species dependent secretion of substrates in addition to substrate and systems level interaction. The most recent computational resource for identifying novel substrates is called BastionX. It has predictors for the secretion systems T1SS-T4SS and T6SS and calculates probabilities of secreting a given protein by each system. Some proteins seem to fit profiles with the best possible score using this method as substrates of at least one secretion system (Wang *et al.*, 2021).

Bacteria must have mechanisms in place to carefully control secretion in order to produce smooth and effective interaction mechanisms, and we expect to be able to properly respond in case a system fails. In this review, the effects of secretion systems crosstalk have been emphasised to show potential consequences of both harmonious and antagonistic action. It is obvious that specific TXSSs' activities have an impact on how well other secretion systems function. One potential effect of a dialogue is compensatory behaviour among secretory systems to attain collective goals in light of the impacts. It is a frequent misconception that a secretion system only carries one protein (Green & Mecsas, 2016). Bacteria likely modify these underutilised systems to transport additional substrates with a similar structure to their principal substrates. Therefore, it is essential to develop methods to study the full-circle circuitry of protein secretion. Outputs will reveal data required to understand the complexities behind secretion system overlap and implications in crucial areas like the use of secretion system inhibitory strategies for disease control.

Implications for crop protection

Advances in disease management have identified virulence regulatory systems such as quorum sensing and secretion systems inhibition as targets. Considerations of T3SS inhibitors to block effector delivery to manage crop disease have been reviewed recently (Pendergrass & May, 2019, Yuan *et al.*, 2020, Sharma *et al.*, 2022). In successful pathogen control, crosstalk would be profitable where targeting one system indirectly targets another system that is interacting with the targeted system. However, this will serve the purpose of effective pathogen control if all interacting systems and their interactive effects are additive, especially in the case where the system directly targeted is responsible for only a part of the detrimental phenotype. A pitfall is observed where effective compounds have been isolated, but their effect is not guaranteed across a broad spectrum of target species (Fan *et al.*, 2019, Puigvert *et al.*, 2019, Hu *et al.*, 2022). Also, for any inhibition strategy to be effective, major virulence determinants of a species must be identified because not all virulence factors are secreted via a single system. Granted, targeting one system may weaken the pathogen but in the advent of potential substrate overlap crosstalk, bacteria will continue to develop resistance, and eventually isolated targeting of secretion systems will not suffice. In addition to OMVs, this can be challenging in instances as in the partial functional redundancies observed between *xpsD* and *xcsD* secretin homologs of *X. campestris* pv. *vesicatoria* T2SSs (Szczesny *et al.*, 2010), and contradicts that there is conserved substrate delivery or roles for secretion systems (Solé *et al.*, 2015). Therefore, targeting regulators of secretion systems may serve as a lucrative alternative, although we still face a challenge in that regulatory crosstalk also lacks commonalities, as exemplified by the unique T6SS integration to varied species-dependent regulatory networks (Leung *et al.*, 2011).

Prediction and screening of secretion system substrates

Secreted proteins and their functions delineate what a secretion system is used for (Filloux, 2022). A portion of secretomes typically studied *in vitro* includes effectors that facilitate interactions between producing bacteria and their hosts. Bioinformatics, proteomics, and biochemical approaches are used to study secretomes. In addition, databases such as Bacterial Secreted Effector Protein DataBase (SecretEPDB) harbour pre-calculated knowledge bases of reference effectors (An *et al.*, 2017).

Computational pipelines use the available secretion systems substrate data (sequence features e.g., secretion signals and conserved motifs) to predict candidate effectors from available microbiota genomes and proteomes. To date, over 40 bioinformatics tools are available to predict GNB T1SS-T9SS substrates (Hui et al., 2021). Bioinformatic tools often rely on two strategies: sequence similarity to known effectors and identification and analysis of unique gene islands of a pathogen. However, there is a limited number of experimentally validated secretion systems cargo, and the poor specificity of prediction tools poses significant challenges to the similarity and unique island predictions' efficiency (Hui et al., 2021, Ma et al., 2014). Poor specificity is partly due to the construction of some algorithms and tools for specific bacteria or substrates (Hui et al., 2021). Another limitation of *in silico* screening of effectors is that most predictors are designed to screen proteomes for substrates of a single secretion system, further limited by a bias towards specific effector groups. Hui and co-workers (2021) presented 45 representative single secretion system substrate predictors. This group is populated with machine learning algorithm based on T3SS and T4SS predictors. Recent advances seek to delimit computational prediction limitations by constructing tools that predict effectors for several secretion systems. One recent semi-knowledge based multiple secretion systems substrate predictor is PREFECTOR (Dhroso et al., 2018). The most recent computational resource for screening for novel TXSS secreted substrates is BastionX (Wang et al., 2021). It has substrate predictors for the secretion systems T1SS-T4SS and T6SS and calculates probabilities of each system secreting a given protein. The upside of this tool is that it predicts both homologs of experimentally validated effectors and novel distant candidates (Wang et al., 2021). Computational pipelines have been utilised to some degree to predict host-pathogen— effector triggered susceptibility agents and pathogen associated molecular patterns or effector triggered immunity protein interactions (Mishra et al., 2019). Developing predictors with broad prediction capabilities will go a long way in screening GNB proteomes for candidate effectors. Bioinformatic approaches used alongside approaches such as proteomics and phage display technologies take the screening process for candidate effectors a step further. Mass spectrometry coupled with bioinformatics is used to identify and annotate proteins in secreted protein sample preparations, thus further filtering the *in silico* predicted candidates and identifying secretomes unbiasedly. Phage display affinity screening identifies proteins implicated in interactions of microbes with their

environments or hosts through virion facilitated physical interactions with target proteins (Gagic *et al.*, 2016). In our view, these platforms offer a means to identify, functionally annotate proteins, and establish protein-protein interaction networks between self (in this case, secreted effectors of multiple systems) and environmental or host proteins. The networks might give insight into probable functions and functional links of proteins secreted by different systems that would guide downstream undertaking applications i.e., disease management. To study substrate cooperation or co-secretion by OMVs, T1SS-T4SS, and T6SS, reporter-based assays can be used. Guided by the secretion systems under inspection, substrates would be cloned as fusions with detectable tags that do not impact secretion (i.e., neutral tags) and expressed by transformed strains for selective identification using affinity chromatography, fluorescence microscopy, enzymatic assays, or Western blot from the wild type and single or double secretion system inactive strain secretomes. Tags associated with an enzymatic activity provide quantitative data on secretion. Densitometry can be used to quantify relative band intensities (Maffei *et al.*, 2017). The final step would be to seek experimental evidence to support the proposed biological roles of the secreted proteins as effectors.

Conclusion

We have emphasised that secretion systems can be interconnected and, in some cases, interdependent through physical contact or by delivering molecular payloads—like effectors and other key proteins into target cells. By retaining such an elaborate capacity to enable interactive system communication in their cells partly helps us explain why bacteria can thrive in their natural habitats through a number of functions; for one, bacteria, among many roles, could derive an agile response to cope with a dynamically changing environment. In other words, this phenomenon could be behind some of the complex phenotypes commonly encountered when multigene pathways are involved, such as bacterial resilience to stress and antimicrobial agents. Be that as it may, this review in no way implies that any of the discussed interactions or their effects serve as a universal mechanism for bacteria to interact with one another or with their hosts. The review only seeks to highlight the magnitude of any beneficial or harmful effects resulting from interactions amongst secretion systems and the potential consequences as these interactions evolve. The absence of empirical data for a crosslink between these secretion systems represents a significant barrier to

analyse their effects in bacteria. Therefore, it is envisioned that taking advantage of the complexity of these linked operations holds out the prospect of new horizons to be explored, such as new treatment strategies for complex bacterial diseases in animals or new plant improvement prospects.

New direction

Considerable efforts are necessary to unpack the molecular mechanisms underlining the convergent function of secretion systems, which may open up new possibilities. As this area is one of a few enigmas in bacterial interactions, research behind the system interaction is likely to gain traction, and a broader consequence of cooperative or antagonistic behaviour that is primarily directed at maintaining adaptability in bacterial responses in the natural environment would emerge. Such details could present an attractive model for developing broad spectrum or multisecretion system antivirulence compounds to alleviate the negative consequences of bacteria, or simply to study the well-coordinated phenotypic developments in prokaryotic species.

Justification of study

As previously indicated, *Pectobacterium* and *Dickeya* spp. are important soft rot bacteria with severe economic consequences. However, currently there is limited knowledge on how pathogens in these genera utilise certain secretion systems to cause infections in their host plants. In *Pbr1692*, one of the highly virulent strains of *Pectobacterium* spp., the production of membrane vesicles and their types and cargo has not been studied, and the role of the T6SS in virulence is elusive (Davidson *et al.*, 2013). Thus, the role they play in secretion systems interplay and, consequently, virulence or survival of the strain is unknown. On the other hand, for nearly all the secretion systems, the complete list of substrates is unavailable, but for some, substantial information on substrate leader/secretion signal motifs is available (Hui *et al.*, 2021). We hypothesise that non-classical secretion, in this case OMVs or the T6SS (given its non-established substrate signature motif), is partly responsible for the exploding aggressiveness of *Pbr1692*. Given the genomic and transcriptomic data available for *Pbr1692*, using proteomics coupled with bioinformatics provides a stratified multi-omics approach to increase the knowledge of the biology of *Pbr1692* (Kwenda *et al.*, 2016, Bellieny-Rabelo *et al.*, 2019). Therefore, in this study, we aimed to explore the characteristics of OMVs, a known non-classical secretion system, and

the versatile T6SS to delineate the roles they might play in protein secretion and the biology of *Pbr1692*.

Study objectives

The main objective of this study was to employ proteomics analysis to identify the proteins translocated by the OMVs and T6SS and the functions associated with them.

The specific objectives were:

1. To isolate, identify, and characterise *Pbr1692* OMVs.
2. To computationally predict the functional annotations of OMV associated proteins.
3. To computationally predict substrates of the type 1-type 4, and type 6 secretion systems of *Pbr1692*.
4. To perform proteomic analysis in order to identify and characterise proteins associated with the T6SS.

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Figure and Table

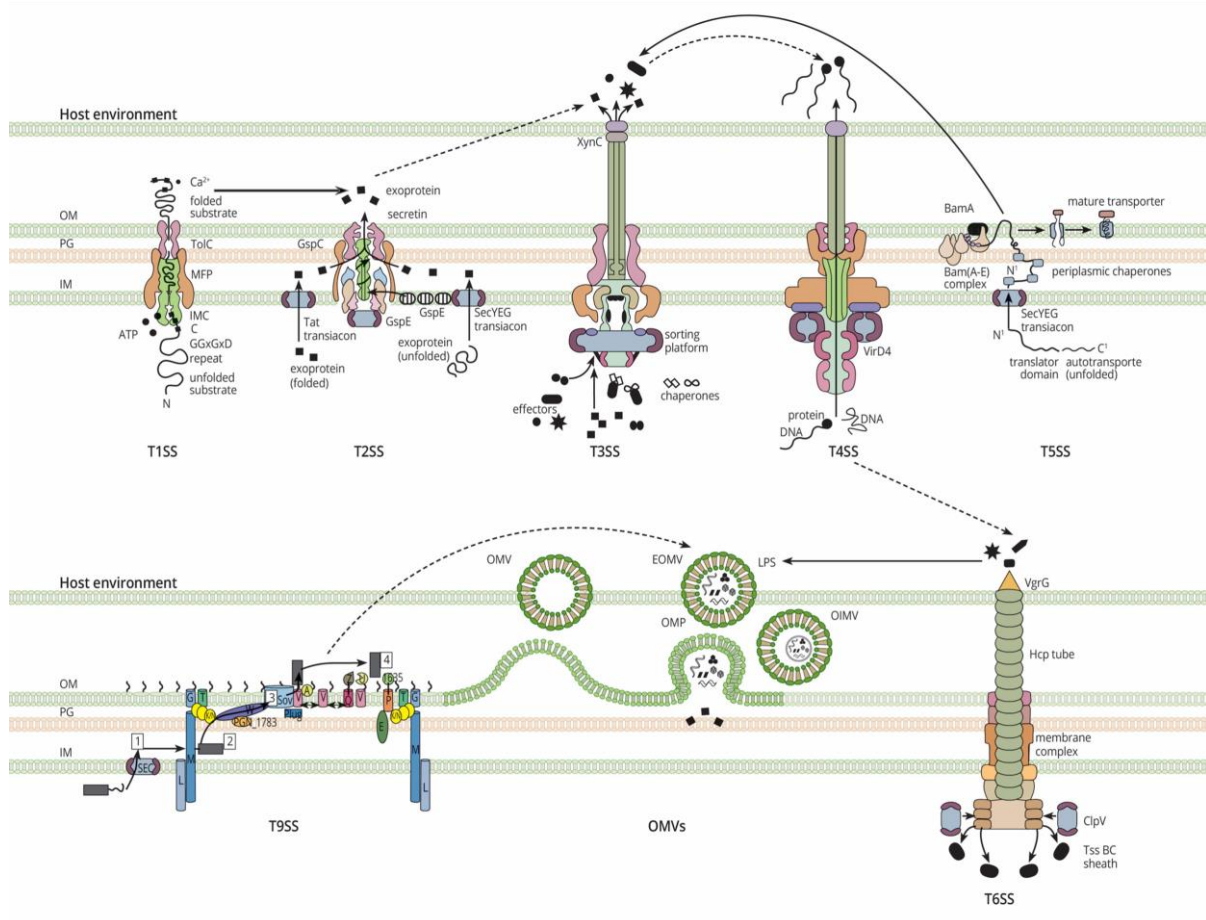


Figure 1.1: Secretion systems (type 1-type 6 secretion system) of Gram-negative bacteria and available evidence of crosstalk with other secretion systems. The figure shows the six secretion systems of GNB (T1SS-T6SS) and the dialogs they are involved in with other secretion systems. The dotted lines indicate interaction at both substrate and system levels, while solid lines indicate interaction at either substrate or system level.

Table 1.1. Interactions among secretion systems in bacteria.

Interacting system(s) ^a	Level of interaction ^b	Outcome and mechanism of interaction ^c	Species	Ref(s)
T3SS-T5SS	Substrate level interaction (substrates: Tir and intimin)	Cooperative. Translocated Intimin Receptor (Tir) acts as a surface receptor for intimin, a T5SS substrate, owing to T3SS-mediated translocation of Tir.	Enteropathogenic <i>Escherichia coli</i>	Kenny <i>et al.</i> , 1997
T3SS-T4SS	Substrate level (BadA) and system level (T4SS) interaction	Antagonistic. BadA's effective length creates a physical barrier between the pathogen and the host cell membrane, preventing T4SS activity.	<i>Bartonella henselae</i>	Lu <i>et al.</i> , 2013
T2SS-T3SS	Substrate level (PCWDEs) and system level (T3SS) interaction	Cooperative. T2SS releases PCWDEs that alter plant cell wall integrity, thereby permitting T3SS-dependent effector protein translocation through.	<i>Xanthomonas citri</i> pv. <i>vesicatoria</i>	Szczesny <i>et al.</i> , 2010
T1SS-T2SS	Substrate level interaction (substrates PrtA and Pell2)	Cooperative. T1SS substrate PrtA post translationally modifies T2SS substrate Pell-2 to Pell-3	<i>Dickeya dadantii</i>	Shevchik <i>et al.</i> , 1998
T4SS-T6SS	Substrate and system level interaction.	Cooperative. T4SS T4P promotes contact-dependent killing by T6SS by bringing adjacent prey closer to the attacker.	<i>Pseudomonas aeruginosa</i> ; <i>Neisseria cinerea</i>	Ho <i>et al.</i> , 2013; Custodio <i>et al.</i> , 2021
T0SS-T6SS	Substrate level interaction (substrates: TseF and TeoL)	Cooperative. T6SS recruits T0SS (outer membrane vesicles or OMVs) either through its effector Tsef (<i>Cupriavidus necator</i>) or TeoL effector and PQS present in <i>Pseudomonas aeruginosa</i> OMVs.	<i>Pseudomonas aeruginosa</i> ; <i>Cupriavidus necator</i>	Lin <i>et al.</i> , 2017; Li <i>et al.</i> , 2021

T0SS-T9SS	Substrate and system level interaction	Cooperative. T9SS effectors, typically extracellular cysteine proteases (gingipains), are anchored on outer membrane vesicles through anionic lipopolysaccharide (A-LPS) moiety of <i>Porphyromonas gingivalis</i> OM. This interaction results in a virulence coat contributing to <i>P. gingivalis</i> infections.	<i>Porphyromonas gingivalis</i>	Veith et al., 2021
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^a Type X secretion systems, where X stands for any number from 1-10, with outer membrane vesicles designated as a T0SS.

^b Full names of molecules and components: BadA, *Bartonella* adhesin A; Tir, Translocated Intimin Receptor; TseF, T6SS effector of Fe uptake; TeoL, T6SS effector for recruitment of OMVs via lipopolysaccharide (LPS); PCWDEs, plant cell wall degrading enzymes; PQS, *Pseudomonas* quinolone signal; T4P, T4SS pilus. 'Substrate' refers to any molecule released by TXSSs (e.g., effectors, adhesins, etc.).

^c The term "outcome" here refers to the way systems interact, which can be either synergistic or antagonistic.

CHAPTER TWO – Isolation and characterisation of outer membrane vesicles of *Pectobacterium brasiliense* 1692

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This chapter has been accepted for publication in the peer reviewed journal, Microorganisms. It is accessible under doi.org/10.3390/microorganisms9091918. I conceptualised the study with Prof. L.N Moleleki. I carried out the experimental work and liaised with external services. Prof. L.N Moleleki helped to shape the initial draft and final manuscript, secured funding for the study, and oversaw the project.

Abstract

Pectobacterium brasiliense (*Pbr*) 1692 is an aggressive phytopathogen affecting a broad host range of crops and ornamental plants, including potatoes. Previous research on animal pathogens, and a few plant pathogens, revealed that outer membrane vesicles (OMVs) are part of Gram-negative bacteria's (GNB) adaptive toolkit. For this reason, OMV production and subsequent release from bacteria is a conserved process. Therefore, we hypothesised that OMVs might transport proteins that play a critical role in causing soft rot disease and in the survival and fitness of *Pbr*1692. Here, we show that the potato pathogen, *Pbr*1692, releases OMVs of various morphologies in Luria Bertani media at 31 °C. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) confirmed the production of OMVs by *Pbr*1692 cells. Transmission electron microscopy showed that these exist as chain-, single-, and double-membrane morphologies. Mass spectrometry followed by gene ontology (GO), clusters of orthologous groups (COGs), virulence factor (VF), carbohydrate-active enzymes (CAZymes), antibiotic resistance ontology (ARO), and Bastion6 type 6 secreted effectors (T6SEs) annotations identified 129 OMV-associated proteins with diverse annotated roles, including antibiotic stress response, virulence, and competition. *Pbr*1692 OMVs contributed to virulence in potato tubers and elicited a hypersensitive response in *Nicotiana benthamiana* leaves. Furthermore, *Pbr*1692 OMVs demonstrated antibacterial activity against *Dickeya dadantii*.

Keywords: Outer membrane vesicles (OMVs), *Pectobacterium brasiliense* 1692, virulence, competition

Introduction

Soft Rot *Pectobacteriaceae* (SRP) are Gram-negative bacteria (GNB) responsible for annual crop losses amounting to millions of dollars worldwide. *Dickeya* and *Pectobacterium* spp. are the primary and most damaging soft rot pathogens causing wilting, blackleg, and tuber decay of potatoes (*Solanum tuberosum* L.), other vegetables, and ornamental plants (Charkowski, 2018, Zaczek-Moczydłowska *et al.*, 2019). First reported in Brazil, *Pectobacterium brasiliense* is not only found in different parts of the world but has also become a significant threat to the production of economically important crops globally (van der Merwe *et al.*, 2010, Onkendi & Moleleki, 2014, Van der Wolf *et al.*, 2017). *Pbr*, like other soft rot bacteria, exhibits many determinants that ensure their persistence in the environment and expansion into new territory and hosts. Similar to other SRP, the main virulence determinants are consignments of plant-cell-wall-degrading enzymes (PCWDEs), such as pectinases, cellulases, and proteinases, which make the genus effective in macerating plant tissue (Chang *et al.*, 2014). Apart from PCWDEs secreted via the type 2 secretion system (T2SS), SRP have an arsenal of virulence factors that elevate pathogenesis. These include iron acquisition, quorum sensing, fimbriae, flagella and motility, polysaccharides, and bacterial secretion systems (T1-, T3-, T4-, T5-, and T6SS), with outer membrane vesicles (OMVs) considered a bacterial type 0 secretion system (Fukuoka *et al.*, 1992, Yaganza *et al.*, 2004, Davidsson *et al.*, 2013, Chang *et al.*, 2014, Guerrero-Mandujano *et al.*, 2017, Piotrowska *et al.*, 2020). Indeed, the role of secretion systems in the coordinated interaction of bacteria with their environment, target hosts, and other bacteria is pivotal (Green & Meccas, 2016). These systems release many proteinaceous and chemical substances for virulence and persistence from the cell cytosol to the extracellular milieu, neighbouring cells, and host cells. Extensive studies of T1SS-T6SSs have determined their composition and the substances they deliver (Costa *et al.*, 2015).

In general, membrane vesicles (MVs) form in GNB by pinching off the outer membranes (OMs), resulting in OMVs and Gram-positive bacteria (GPB) by disrupting the peptidoglycan layer forming microvesicles (Woith *et al.*, 2019). Owing to their essential role in transferring bioactive molecules from donor to recipient cells, MVs, particularly OMVs in GNB, are ubiquitous and conserved (Yaganza *et al.*, 2004,

Guerrero-Mandujano *et al.*, 2017, Piotrowska *et al.*, 2020). Although models of the mechanisms of biogenesis, cargo, and functions of OMVs continue to be reported, not much is known about the mechanism of loading and sorting of the cargo into the vesicles for transportation (Schwechheimer & Kuehn, 2015, Toyofuku *et al.*, 2019). Outer membrane-derived OMVs are enriched in periplasmic proteins, while outer-inner membrane vesicles (O-IMVs) have inner and outer membranes enriched in addition to periplasmic proteins and cytoplasmic contents. More so, Explosive OMVs (EOMVs) carrying cytoplasmic proteins in addition to periplasmic proteins have been reported (Roier *et al.*, 2016, Turnbull *et al.*, 2016, Toyofuku *et al.*, 2019). Studies also report OMVs of larger sizes, aggregates, chains, and tubes (Li *et al.*, 2016). However, there are no baselines for isolation methods; hence, the types, cargo, and specific functions of OMVs will vary from one species to another, making comparisons difficult (Orench-Rivera & Kuehn, 2016, Reimer *et al.*, 2021). We have yet to learn more about the species and strain-specific roles of OMVs of phytopathogenic bacteria in bacteria-bacteria and bacteria-host interactions, as well as their potential to control plant diseases.

So far, we know that OMVs have physiological, protective, and adaptative roles in the environment (Avila-Calderón *et al.*, 2015, Haurat *et al.*, 2015). Phytopathogenic bacteria produce OMVs involved in colonisation, virulence, host immune elicitation or evasion, defence neutralisation, and biofilm formation (Ionescu *et al.*, 2014, Bahar *et al.*, 2016, Katsir & Bahar, 2017). Therefore, bacterial cells communicate with each other and the environment through OMVs. This form of communication is without energy expenditure to transfer bacterial messenger molecules, toxins, and nutrient acquisition/scavenging systems that consequently affect the environment even though cargo sorting into and producing OMVs is likely resource depleting (Schwechheimer & Kuehn, 2015, Woith *et al.*, 2019). Moreover, the rapid release of toxins and misfolded proteins produced under stressful conditions, surface binding and antimicrobial agents, and hydrolytic enzymes ensures the survival of the producing bacteria (Li *et al.*, 2016). Unlike other secretion mechanisms that solely transport soluble material, OMVs enable bacteria to secrete insoluble molecules complexed with soluble material (Kulp & Kuehn, 2010). Since they transport a set of biological macromolecules typifying most secretion systems' substrate, OMVs play a critical role as the GNB secretion systems' succour system. Therefore, bacterial OMVs provide relief from the

secretion burden to delivery systems (Guerrero-Mandujano *et al.*, 2017). OMVs ferry lipopolysaccharides (LPS), phospholipids, peptidoglycan, outer membrane proteins (OMPs), cytoplasmic proteins, nucleic acids, ion metabolites, and signalling molecules as part of their structure and in their lumen for various destination outcomes (Koeppen *et al.*, 2016, Vanaja *et al.*, 2016). External surface association of toxins and nucleic acids with OMVs also exists (Bitto *et al.*, 2017). Upon reaching the targeted destination, OMVs release their cargo, which may be involved in different biological roles (Kuehn & Kesty, 2005, Haurat *et al.*, 2015). Exploration of OMV cargo in other GNB points to several such roles, including increased virulence, immune activation or suppression, biofilm formation, inter- or intraspecies and interkingdom delivery of defensive and offensive molecules, respectively, uptake, and pathogen adherence or detachment (MacDonald & Kuehn, 2013, Ionescu *et al.*, 2014, Jan, 2017, Roszkowiak *et al.*, 2019). For example, Enterotoxigenic *Escherichia coli* (ETEC) vesicles are essential in disseminating heat-labile enterotoxin to host cells during bacterial infection leading to diarrhoea (Horstman & Kuehn, 2000). The OMV-enriched proteome of two human microbiota, *Bacteroides fragilis* and *Bacteroides thetaiotaomicron* showed high glycosidases and proteases, including those active *in vitro* (Elhenawy *et al.*, 2014). Moreover, proteomics has identified different putative biologically active molecules like nucleotides and immunogenic peptidoglycan (Kroniger *et al.*, 2018). OMV proteins are involved in communication, nutrient acquisition, stress responses, and virulence (Avila-Calderón *et al.*, 2015). Some of the identified OMV-specific proteins in previous studies are uncharacterised hypothetical proteins; hence, it may be challenging to predict functions for these or fully understand the proteins found in the vesicles (Berleman *et al.*, 2014).

Pectobacterium brasiliense 1692 is more virulent than other *Pectobacterium* spp. and is able to outcompete other members of SRP under different environmental conditions (Marquez-Villavicencio *et al.*, 2011, Shyntum *et al.*, 2019). We presupposed that OMVs could contribute to the delivery of PCWDEs, toxins, and effectors involved in either virulence or competition. In this work, we established that *Pbr*1692 naturally releases MVs throughout its growth. These OMVs could macerate potato tuber tissue, elicit a hypersensitive response (HR) on *Nicotiana benthamiana* leaves, and were able to inhibit the growth of another SRP, *D. dadantii*. We carried out proteomics analysis of isolated vesicles to identify the OMV-associated proteins. Functional annotations

showed that diverse functions could be ascribed to *Pbr1692* OMVs centralised in virulence and fitness.

Materials and methods

The strains and growth conditions

Pectobacterium brasiliense 1692 and *D. dadantii* (with plasmid pMP7605 conferring gentamycin resistance) were cultured in Luria Bertani (LB) media at 31 and 32 °C, respectively, with shaking at 120 rpm for 12-14 h. Where required, for the growth of *D. dadantii*, growth media were supplemented with 15 µg/mL gentamycin antibiotic (Sigma-Aldrich, St. Louis, MO, USA).

Scanning electron microscopy analysis of cells

Five millilitres of *Pbr1692* cells in exponential and stationary phase were pelleted at 3000 rpm for 3 min. The supernatant was discarded, and then cell pellets were washed with 0.1 M phosphate buffer pH 7.4 on a rotator and after the fixation and post-fixation steps thrice for 10 min. First, the cells were resuspended in 2.5% glutaraldehyde fixative for 30 min, post-fixed with 1% osmium tetroxide (w/v) for 30 min, and then dehydrated by a graded series of ethanol (30%, 50%, 70%, and 90%) for 10 min. The cells were then dehydrated by 100% ethanol twice for 10 min and once for 40 min. Next, the cells were pelleted and embedded in hexamethyldisilazane (HMDS):ethanol mixture for 1 h twice. Following this, fresh HDMS was added, and then the cells were left to dry. The cells were then mounted and coated with carbon for visualisation using the Zeiss Crossbeam 540 Field Emission-Scanning Electron Microscope (FE-SEM) (Zeiss, Oberkochen Germany) operated at 1.00 kV.

Transmission electron microscopy analysis of cells

Five millilitres of *Pbr1692* culture in the stationary phase were pelleted at 3000 rpm for 3 min. The supernatant was discarded, and then the cell pellet was washed with phosphate buffer on a rotator and after the fixation and post-fixation steps thrice for 10 min each time. The cells were resuspended in 2.5% glutaraldehyde fixative for 30 min and then post-fixed with 1% osmium tetroxide (w/v) for 30 min. After that, a graded series of ethanol (30%, 50%, 70%, and 90%) was used to dehydrate the cells for 10

min. Dehydration by 100% ethanol was done twice for 10 min and once for 40 min. Dehydrated cells were mixed with epoxy resin:ethanol mixture for 1 h twice. Fresh epoxy was added, and then the cells were dried in an oven for over 24 h for polymerisation. The embedded sample was trimmed, sectioned, and stained for visualisation using the transmission electron microscope (TEM) (JOEL JEM 2100F, JOEL Ltd. Tokyo, Japan).

Isolation of OMVs

Outer membrane vesicles were isolated at the stationary growth phase of *Pbr1692* (OD₆₀₀ of ~1–1.2) according to (Altindis *et al.*, 2014) and (Davies *et al.*, 2019) with modifications. First, one litre of the *Pbr1692* culture was subjected to low-speed centrifugation at 16,000× g for 20 min at 4 °C to pellet the cells. After that, cell debris and macromolecules were removed by high-speed centrifugation at 38,000× g for 1 h at 4 °C. The cell-free supernatant was filtered using a 0.22 µm bottle filter top under vacuum to remove residual cells (Merck, Darmstadt, Germany). LB agar plates were inoculated with 200 µL of cell-free filtrates and incubated at 31 °C for 48 h to ensure total removal of bacterial cells. The cell-free supernatants were reduced 100-fold by concentration in Amicon® Ultra-15 (MWCO 30 kDa) centrifugal units (Merck, Darmstadt, Germany). OMVs were pelleted from the concentrated supernatant in an SW 55 Ti Rotor (Beckman Coulter, Brea, CA, United States) at 145,000× g at 4 °C for 6 h and then resuspended in phosphate buffered saline (PBS) (Sigma-Aldrich, St. Louis, MO, USA). OMVs for virulence assays were washed twice in the SW 55 Ti Rotor at 145,000× g at 4 °C for 6 h. Protein concentration was checked using the nanodrop at A₂₈₀. OMV samples were stored at –20 or –80 °C after freezing in liquid nitrogen.

Negative staining of OMVs

Purified OMVs were spotted on carbon-coated grids for adsorption for 5 min. The vesicles were, after that, negatively stained with 1% (w/v) uranyl acetate for 3 min. Finally, the OMVs were visualised using TEM (JOEL JEM 2100F, JOEL Ltd. Tokyo, Japan).

Nanoparticle tracking analysis and Bradford assay of OMVs and cell-free supernatants

Isolated OMV samples and *Pbr1692* cell-free supernatants were analysed using the NanoSight NTA version 3.3 (Malvern Panalytical, Malvern, UK) to determine nanoparticle concentration and distribution. Cell-free supernatants were prepared for NTA according to (Ionescu *et al.*, 2014) with slight modifications. Three biological replicates of *Pbr1692* cells were grown to the exponential (OD_{600} of ~ 0.5) and stationary growth phase (OD_{600} of ~ 1.0). Cells were pelleted out of culture using low-speed centrifugation at $16,000\times g$ for 20 min twice at $4^{\circ}C$. The cell-free supernatants were filtered under vacuum to remove residual bacterial cells. A test volume of 1.5 mL of each replicate was injected into the NTA and analysed in triplicate (each run = 30 s video). The videos were captured and processed using NTA 3.3 Dev Build 3.3.104 (Malvern, Panalytical, Malvern, UK). In between runs, samples were advanced to introduce fresh sample aliquots for quantification. The camera sensitivity and detection threshold were optimised per video, and the temperature was set between 20 and $22^{\circ}C$. Readings were analysed using the NTA software v3.3. LB media were used as a blank. The Bradford reagent was used to determine the protein concentration of the cell-free supernatants (Bio-Rad Laboratories, Hercules, California, United States) using bovine serum albumin (BSA) as a standard (Sigma-Aldrich, St. Louis, MO, USA).

Protein extraction and digestion

Outer membrane vesicle proteins of three biological replicates were extracted according to the BGI Tech Solutions Co., Ltd. (Hong Kong, China) protocol. SDS at 4% was added to the OMVs and then the samples were boiled for 5 minutes to extract the proteins. Proteins were purified by gel electrophoresis and then the gel was soaked in 50 μL pure water for 30 min. Gel strips were cut and put in separate tubes. The samples were decolorised in 50 mM ammonium bicarbonate (NH_4HCO_3): acetonitrile (ACN) mixture (v/v) at $37^{\circ}C$ for 30 min, then centrifuged and dried. The process was repeated until the blue dye faded. The gels were dehydrated using 500 μL acetonitrile twice and then left to air dry. The gels were then covered in 10 mM dithiothreitol (DTT) (1M DTT: 25 mM NH_4HCO_3) and incubated at $56^{\circ}C$ for 1 h. After that, the samples were left to cool to room temperature and soaked with 55 mM iodoacetamide (IAM) (0.55 M IAM: 25 mM NH_4HCO_3) and kept in the darkroom at room temperature for 45 min. The samples were thereafter washed twice with discolourising solution and then

with pure water before adding 500 μ L acetonitrile. The samples were then vortexed for 5 min and left to dry thoroughly. The dry gel samples were digested by covering them in enzyme solution diluted to 0.01 μ g/ μ L with 25 mM NH_4HCO_3 for 30 min at 4 °C and then incubating in buffer overnight at 37 °C. Five times volume of 50% ACN were added to the samples before vortexing for 5 min and centrifuging at 5000 $\times$ *g* for 1 min. The supernatants were collected into fresh tubes and 5 times 100% ACN was added. The samples were vortexed for 5 min and centrifuged at 5000 $\times$ *g* for 1 min. After, the supernatants were transferred into fresh tubes and then centrifuged at 25,000 $\times$ *g* for 5 min before the samples were freeze-dried.

High performance liquid chromatography

High performance liquid chromatography was performed according to the BGI Tech Solutions Co., Ltd. (Hong Kong, China) protocol. The freeze-dried peptide samples were reconstituted with mobile phase A (2% ACN, 0.1% formic acid (FA)) and then centrifuged at 20,000 $\times$ *g* for 10 min. The supernatant was taken for injection and separated by a Shimadzu LC-20AD model nanolitre liquid chromatograph. The sample was first enriched in the trap column and desalted and then channelled into a tandem self-packed C18 column (75 μ m internal diameter, 3 μ m column size, 15 cm column length). Separation was at a flow rate of 300 nL/min by the following effective gradient: 0–6 min, 6% mobile phase B (98% ACN, 0.1% FA); 6–40 min, mobile phase B linearly increased from 6% to 25%; 40–48 min, mobile phase B rose from 25% to 40%; 48–51 min, mobile phase B rose from 40% to 90%; 51–55 min, 90% mobile phase B; 55.5–60 min, 6% mobile phase B. The nanolitre liquid phase separation end was directly connected to the mass spectrometer.

Electrospray ionisation mass spectrometry

Liquid phase chromatography-separated peptides were passed to the ESI tandem mass spectrophotometer, TripleTOF 5600 (SCIEX, Framingham, MA, USA) according to the BGI Tech Solutions Co., Ltd. (Hong Kong, China) protocol. Nanospray was used as an ion source (SCIEX, Framingham, MA, USA), and the emitter was a needle (New Objectives, Woburn, MA, USA) drawn from quartz material. The specific applied parameters of ion spray voltage 2300 V, nitrogen pressure 35 psi, spray gas 15, and spray interface temperature 150 °C were set. Scanning in high sensitivity mode, the

cumulative scan time was 250 ms, and the scan quality range was between 350 and 1500 Da. Based on the MSI scanning information and the ionic strength in the MSI spectrum from high to low, the first 30 ions that exceeded 150 cp were fragmented, and the MS2 information was scanned. The screening was done according to the following criteria: (a) the m/z range was 350–1250 m/z ; (b) the number of charges was 2–5 charges; (c) the dynamic elimination of the parent ion was set to half of the peak time (~12 s); and the fragmentation of the same parent ion did not exceed 2 times. The scan accumulation time MS2 mass spectrum was 50 ms. The collision energy was set to 'Rolling Collision Energy'.

Database search and quantification

The MS/MS experimental data were aligned with theoretical MS/MS data from a *Pbr* database (14096 sequences). First, the MS/MS raw data were converted to an *.mgf file and searched for matches in the database using Mascot software (Matrix Science, London, UK; v2.3.02). A fragment mass tolerance of 0.1 Da and a peptide tolerance of 0.05 Da were set. Carbamidomethylation cysteine (C) was the fixed modification. The oxidation of methionine (M), conversion of N-terminal glutamine (Gln) to pyroglutamic acid (pyro-Glu), and the deamination of N-terminal glutamine (Q) were set as variable modifications. Trypsin specificity was set to allow 1 missed cleavage. The search results were rescored using Percolator to improve the matching accuracy. Then, the output was filtered by a 1% false determination rate (FDR) at the spectral level (PSM-level FDR $\leq$ 0.01) to obtain the significant identified spectrum and peptide list. Proteins were linked to peptides, and a series of protein groups were generated. The intensity-based absolute protein quantification (iBAQ) was carried out as previously described (Schwanhäusser *et al.*, 2011).

Sequence analysis and annotations

The protein sequences were first retrieved from the UniProt database to establish their functional annotations. The gene ontology (GO) term claims for the proteins were assigned using the GO international standard gene function classification system (Consortium, 2019). The GO functional annotation included protein2go and go2protein. The subcellular localisation of each identified protein was predicted using PSORTb v3.0.3 (Yu *et al.*, 2010). Protein classification was carried out using the

clusters of orthologous groups of proteins system (COG) (Galperin *et al.*, 2021). Virulence proteins were identified using the Virulence Factor DataBase (VFDB) (Liu *et al.*, 2019). Carbohydrate-active enzyme (CAZyme) annotation was performed using dbCAN2 (Zhang *et al.*, 2018). We also used a Comprehensive Antibiotic Resistance Database (CARD) to identify proteins essential for the persistence of *Pbr1692* in the presence of antibiotics (Alcock *et al.*, 2020). Type 6 secreted effectors (T6SEs) were predicted using Bastion6 (Wang *et al.*, 2018).

Virulence assays of *Pbr1692* OMVs

Protease activity of OMVs

Twenty micrograms of OMVs were mixed with loading buffer (without β -mercaptoethanol) and incubated at room temperature for 10 min. The samples were then loaded onto a 12% SDS-0.1% gelatin-polyacrylamide gel (v/v) according to Elhenawy and coworkers (2014) with modifications. The gel was run at 150 V (30–50 mA) using a 1X electrophoresis buffer. Gelatinase activation was performed by washing the gel three times with gelatinase renaturation buffer for 20 min each time with agitation. After that, the gel was incubated in a washing buffer for 24 h at 37 °C. For staining, the gel was incubated in Coomassie Brilliant blue G 250 gel staining solution for 1 h at room temperature in a shaker. The gel was destained in the destaining solution until visible clearing was observed.

Maceration of potato tubers

Tap-water-washed *Solanum tuberosum* L. (cv. Mondial, a susceptible cultivar) potato tubers were surface sterilised by soaking in 10% (v/v) sodium hypochlorite (NaOCl) for 10 min. The potatoes were rinsed three times with sterile distilled water and then once with 96% ethanol. The potatoes were then allowed to air dry before 1 cm holes were punched with a sterile tip. The generated wounds were inoculated with 10 μ L OMV suspension in PBS ($\sim 1 \times 10^9$ OMVs/mL), OD₆₀₀ of 1 *Pbr1692* cells, and *Pbr1692* cell-free supernatant. For the negative control, sterile PBS was inoculated. The inoculation sites were then sealed with petroleum jelly, and the potatoes were incubated under humid conditions at 31 °C for 72 h. The lesion sizes were measured at this time. The experiment was carried out using three biological replicates, two independent times.

Nicotiana benthamiana elicitation of hypersensitive response by *Pbr1692* OMVs

Two four-week-old *N. benthamiana* plants were infiltrated by piercing three different leaves per plant with a needle and saturating each leaf with 1 mg/mL OMVs, cell-free supernatant, and OMV-wash supernatant as a control through the puncture two independent times.

Antimicrobial activity of outer membrane vesicles

Frozen stocks of target soft rot bacterium, *D. dadantii* (harbouring pMP7605 which confers gentamycin resistance), and *Pbr1692* were streaked on LB agar plates and incubated overnight at 32 and 31 °C, respectively. Single colonies were picked from the plates to inoculate 20 mL of fresh LB broth and incubated as above with shaking at 120 rpm for 12–14 h. Overnight cultures were then normalised to OD₆₀₀ of 0.5. Twenty microliters of OMV suspension in PBS (~1 × 10⁹ OMVs/mL) and *D. dadantii* (OD₆₀₀ of 0.5) was mixed in a 1:1 ratio and spotted on an LB agar plate and then incubated at 32 °C for 16 h. A volume of 20 µL of *D. dadantii* (OD₆₀₀ of 0.5) cells was also mixed with an equal volume of the normalised *Pbr1692* cells (OD₆₀₀ of 0.5) as a positive control. PBS was used as a negative control. All the spots were scrapped into 1 mL LB post-incubation, serially diluted with sterile water, and then plated on LB plates supplemented with gentamycin. The single colonies were counted to enumerate viable cells in three independent plates two independent times.

Statistical analysis

In this study, experiments were performed in triplicate two independent times. Where applicable, a student *t*-test and analysis of variance using R v3.6.1 were performed to determine statistical significance, and a *p*-value less than 0.05 or 0.01 (*p* < 0.05 or *p* < 0.01) was a statistically significant difference.

Results

Identification of *Pbr1692* OMVs

To establish whether *Pbr1692* produces OMVs in rich media, SEM was used to analyse cells cultured to exponential and stationary growth phases (Figure 2.1A-D). OMVs were observed budding off from or on the surfaces of rod-shaped bacterial cells

both in the exponential and stationary phases. OMV aggregates and biofilm-like matrix structures were observed at both time points (Figure 2.1B, D). In addition, there were distinct areas on the bacterium where OMV clustering signalled where production was concentrated, “hot spots” (white arrows).

Electron microscopy and nanoparticle analysis of vesicles

Pectobacterium brasiliense 1692-pelleted cells were analysed using TEM to observe budding vesicles (Figure 2.2A). The cell density and total protein concentration of exponential and stationary cell-free supernatants were OD₆₀₀ of 0.547 and 1.050 with a total protein of 60 and 102 µg/mL, respectively. However, with no statistically significant difference in nanoparticle concentration or size distribution (Figure S2.1), OMVs were isolated in the stationary phase and analysed using NTA. The OMV particle size distribution of 75–355 nm is shown in Figure 2.2B. The recorded average size was 173 ± 1.9 nm, and the mode diameter was 150.5 ± 7.9 nm.

The isolated OMVs were negatively stained and analysed using TEM (Figure 2.3). Vesicles of different sizes and morphologies were observed. OMVs of varying sizes are shown (Figure 2.3A). Chain-like and aggregated OMVs were also identified (Figure 2.3B). Some of the vesicles had double membranes (Figure 2.3C).

Proteomic analysis of OMVs

Pectobacterium brasiliense 1692 encodes 4135 proteins in its genome (<https://www.uniprot.org/proteomes/UP000464068> accessed on 15 June 2021). The TripleTOF 5600 mass spectrophotometer was used to analyse the digested proteins of OMV samples isolated from three independent cultures to produce 119,240 spectra in total. Mascot analysis of the mass spectrometry generated data by searching against a *Pbr* constructed database was used to identify the OMV proteins. A total of 451 proteins were identified as potential OMV cargo of *Pbr*1692 in OMV-enriched fractions of three independent culture supernatants (Table 2.1).

A cut-off of two or more predicted peptides per identified protein was used to filter the proteins identified in the three biological replicates shown in Table 2.1. In total, 117, 126, and 171 proteins were considered OMV cargo of OMV1, OMV2, and OMV3, respectively (Figure 2.4). A total of 114 (97%) of the 117 proteins identified in OMV1

were present in the other two replicates (Figure 2.4). Of the 126 proteins identified in the sample OMV2, 109 (87%) were present in the other two replicates. Lastly, among OMV3 proteins, 115 (67%) placed in replicate OMV1 and OMV2. OMV1, OMV2 and, OMV3 had 3, 17, and 56 unique proteins, respectively (Figure 2.4A). A total of 129 proteins present in at least two biological replicates were taken for further analysis as *Pbr1692* OMV-associated proteins and cargo (Table S2.1). The biological replicate protein numbers suggest a high degree of consistency and reproducibility. In total, 80 (62%) of the 129 proteins were present in all three replicates, and 49 (38%) were present in two biological replicates. To predict the subcellular localisation of the OMV proteins, PSORTb v3.0.3 was employed. Most of the OMV proteins were outer membrane (28%) and cytoplasmic proteins (28%) (Figure 2.4B). Other locale predictions included cytoplasmic membrane proteins (9%), extracellular proteins (14%), and periplasmic proteins (5%). Subcellular predictions of 16% of the OMV proteins were not assigned subcellular localisations.

The iBAQ was used to establish the most abundant proteins in OMVs. The top 50 most abundant proteins are shown in Table 2.2. Among the most abundant proteins, most were outer membrane proteins. Very few proteins in the top 50 were identified as cytoplasmic membrane (3) and periplasmic (1) proteins. Nine proteins in the most abundant OMV proteins had no localisation information, according to the PSORTb tool. Gene ontologies were also assigned to the most abundant proteins (Table 2.2).

Functional annotation of OMV proteins

Gene ontology (GO), clusters of orthologous groups (COGs), carbohydrate-active enzyme (CAZyme), Virulence Factor DataBase (VFDB) integrated with VFanalyser, antibiotic resistance ontology (ARO), and Bastion6 T6SE annotations were employed to describe the potential functions of the OMV proteins. Proteins were annotated in GO terms to comprehensively provide all possible claims of their properties within the following ontologies: molecular, cellular, and biological process functions. In total, 240 GO annotations were assigned to 104 OMV proteins. The WEGO output (<https://wego.genomics.cn/> accessed on 26 April 2021) was used to visualise the respective ontology entries of the proteins with assigned GO terms within the three ontologies and the number of proteins associated with each group. A total of 81 biological functions and 80 cellular component functions were assigned. Under the

ontology of biological processes, the highest number of proteins was found in annotated cellular (GO:0009987) and metabolic process (GO:0008152) functions. The cellular component terms cell (GO:0005623) and cell part (GO:0044464) represented 59.6% and 58.7%, respectively.

A total of 79 molecular function GO annotations were assigned (Figure 2.5A). In total, 56 (53.8%) of the 104 proteins were assigned catalytic activity functions (GO:0003824). The most abundant in this entry had hydrolase (GO:0016787), transferase (GO:0016740), and lyase (GO:0016829) activity. Proteins exhibiting phospholipase A1 or A2 activity, polygalacturonase activity, peptidase activity, and cellulase activity were among the identified hydrolases. Nine proteins (8.7%), six (5.8%), and three (2.9%) were annotated oxidoreductase activity, catalytic activity acting on a protein, and peptidoglycan muralytic activity, respectively. Forty-two proteins exhibiting binding ability (GO:0005488) made 40.2% of the total number of annotated proteins. Seven (6.7%) proteins were annotated drug binding activity. Other entries in the molecular ontology included structural molecule activity (GO:0005198), transporter activity (GO:0005215), a molecular function regulator role (GO:0098772), and antioxidant activity (GO:0016209). Sets of COGs established more useful classification clues (Figure 2.5B). COGs linked the proteins to an evolutionary trail to clarify the proteins' roles. The cluster with the highest number of proteins included cell wall/membrane/envelope biogenesis groups. Relatively high numbers were also assigned to groups with carbohydrate transport and metabolism, energy production and conversions, and inorganic ion transport and metabolism functions. COG analysis predicted generalised positions for eight proteins and did not establish cluster links for 13 proteins. The other protein cluster groups identified are shown in Figure 2.5B.

Identification of OMV virulence factors, carbohydrate-active proteins, antibiotic agents, and T6SEs

We established further which OMV proteins exhibit virulence, carbohydrate-active, antibiotic resistance, and antibiotic properties (Figure 2.6). Thirty virulence proteins were identified using the Virulence Factor DataBase (VFDB). The identified virulence factors (VFs) included six pectate lyases with carbohydrate metabolism roles, attachment invasion locus protein, adhesin, and ATPase activity. Other VF proteins found had catalase (A0A0M2F307), esterase (EstA) (A0A0M2F3J1), and contact-

dependent inhibition A (CdiA) (A0A0M2EZM6) activity. The elongation factor (EF-Tu) and flagellin were also among identified VFs. Nineteen enzymes important in carbohydrate metabolism were identified. We also identified two F5/8 type C domain-containing proteins, arabinogalactan endo-beta-1,4-galactanase, endoglucanase, and membrane-bound lytic murein transglycosylase each. Other enzymes were peptidoglycan lytic exotransglycosylase, glycoside hydrolase, murein transglycosylase B, penicillin-binding protein, endopolygalacturonase, exo-poly-alpha-D-galacturonidase, and an autotransporter protein. The Comprehensive Antibiotic Resistance Database (CARD) was used to identify proteins involved in bacterial antimicrobial resistance; hence, such proteins are instrumental in survival. Eight proteins, namely, OmpA, OmpK, CeoB, LptD, RspA, SecD, FusA, and TolC, were identified. These eight proteins were predicted to facilitate antibiotic efflux, alter the antibiotic target, and reduce permeability to the antibiotic. LptD was among nine out of 72 proteins we identified with a probability >0.9 to be T6SEs. A phospholipase effector and the avirulence protein, suspected to induce HR in plant hosts, were also identified. Other proteins included porins, a phage tail protein, a YjbH domain-containing protein, and TonB dependent plug domain-containing protein.

Contribution of *Pbr1692* OMVs to virulence and hypersensitive response

Using gelatine as the protease target substrate, zymography validated the protease activity of OMVs. In this regard, OMVs displayed a ~55KDa protein demonstrating gelatine hydrolysis of the gelatine incorporated in the gel shown by the white zone clearing at this site (Figure 2.7A). On the contrary, no activity was observed for denatured/heat inactivated OMVs (negative control) (Figure 2.7A). Further, OMV cargo macerated tissue of susceptible *Solanum tuberosum* L. cv Mondial three days post-inoculation (Figure 2.7B). As expected, the maceration degree was highest when potatoes were inoculated with *Pbr1692* cells as a positive control compared to cell-free supernatant or OMV cargo (Figure 2.7B). OMVs and *Pbr1692* supernatant also elicited HR, visible at 24 hpi. On the other hand, no activity was observed for the OMV supernatant obtained after the second OMV washing step, implying that all loosely associated proteins were successfully removed and the OMV cargo specifically inflicts observed phenotypes (Figure 2.7C).

Antimicrobial activity of OMVs against *Dickeya dadantii*

We explored the possibility that the OMVs carry bacterial growth inhibitory components previously shown to give *Pbr1692* a competitive advantage against other bacteria (Shyntum *et al.*, 2019). OMVs isolated from the *Pbr1692* culture exhibited antibacterial activity against *D. dadantii*, another soft rot pathogen found within the microbial community during potato infection. OMVs reduced the *D. dadantii* cells by approximately 95% (Figure 2.8). As expected, the proliferation of *D. dadantii* was highly inhibited by *Pbr1692* as a positive control compared to when *D. dadantii* was cocultured with PBS as a negative control.

Discussion

The secretion systems of GNB deliver proteins to different host cell compartments and into the external milieu to invade and evade the host, alienate competitors, and maximise resource usage (Green & Mecsas, 2016). However, only a handful of reports contribute to current knowledge of the role of OMVs, specifically those of phytopathogens, in this regard. Since *Pbr1692* is an important phytopathogen of potatoes, we were interested in determining the type of vesicles it produces, the cargo ferried by these vesicles, and the potential roles they may play in the biology of this pathogen. Towards this end, we found that in nutritionally rich media, OMVs naturally emerge from distinct areas of *Pbr1692* cells during *in vitro* growth, suggesting that there are dedicated “hot spots” of membrane blebbing. OMVs do not form spontaneously; hence, “hot spots” may be required to maintain cell viability during vesiculation (Deatherage *et al.*, 2009). Vesicles were also embedded in a biofilm-like matrix and, thus, are possible ‘nucleation’ biofilm centres from which biofilm formation can be centred (Schooling & Beveridge, 2006, Yonezawa *et al.*, 2009, Altindis *et al.*, 2014, Cooke *et al.*, 2020, Seike *et al.*, 2020). In our previous studies, we found that part of the strategy used by *Pbr1692* to invade potato stem xylem tissue is forming biofilms (Kubheka *et al.*, 2013, Moleleki *et al.*, 2017). This study brings new insights into the possible contribution of OMVs towards *Pbr1692* biofilm formation. In the current study, we found that *Pbr1692* produces OMVs and O-IMVs. A similar observation was previously reported for *P. betavascularum* IFB5271 and *P. zantedeschiae* 9M (formerly *P. atrosepticum*) (Piotrowska *et al.*, 2020). The presence of O-IMVs makes it difficult to dismiss cytoplasmic proteins, RNA, and DNA as cell

lysis contaminants in vesicle preparations (Cooke *et al.*, 2019, Piotrowska *et al.*, 2020). Moreover, O-IMV constituents seem sensitive; hence, they are enclosed in a double membrane. O-IMV production is, thus, specialised, possibly through circularizing membranes of dead cell minority entrapping cytoplasmic proteins and nucleic acids (Cooke *et al.*, 2019). In contrast to localised blebbing from “hot spots”, explosive or endolysin-triggered cell lysis is a novel biogenesis mode of EOMVs and O-IMVs containing periplasmic and cytoplasmic proteins (Turnbull *et al.*, 2016, Toyofuku *et al.*, 2019, Piotrowska *et al.*, 2020). There is also sufficient evidence of moonlighting cytosolic proteins and DNA that function on the bacterial cell surface associated with OMVs (Př et al., 2013, Pérez-Cruz *et al.*, 2015). Other membrane vesicle types and extensions are nanopods, nanotubes, and nanowires (Schwechheimer & Kuehn, 2015, Orench-Rivera & Kuehn, 2016). Chain-like OMVs and OMV aggregates were observed in this study. Cell-cell communication bridging is assumed to be the reason for the chain-like phenotype (Li *et al.*, 2016).

A total of 129 proteins associated with *Pbr1692* OMVs was identified. This number compares fairly to other studies, such as one involving *Xylella fastidiosa*, despite differences in culture growth conditions, OMV sizes, and cargo (Altindis *et al.*, 2014, Veith *et al.*, 2014, Yun *et al.*, 2017). The only other study involving *Pectobacterium* spp. vesicles using *P. betavascularum* IFB5271 and *P. zantedeschiae* 9M identified 62 proteins, nearly half the number of proteins identified here (Piotrowska *et al.*, 2020). With regard to cargo, most of the proteins identified were outer membrane (36) and cytoplasmic proteins (36). The latter, in early studies, were considered unexpected OMV cargo since OMVs predominantly carried periplasmic and outer membrane components (Lee *et al.*, 2016). Extracellular proteins (21) were also found in relatively high numbers as part of OMV cargo. This is not surprising since *Pbr1692* directs its extracellular enzymes into the periplasmic space for delivery via the T2SS into the extracellular environment (Charkowski *et al.*, 2012). Therefore, proteins enriched in the periplasm have a higher probability of being loaded into single-membrane vesicles produced by this bacterium.

Among the top 50 most abundant proteins mapped in this study (Table 2.2), there was a high number of cell and cell part annotated proteins. These included outer membrane proteins, which are generally essential as barriers against stressful

conditions. Therefore, this points to a protective role of OMVs to their cargo and producing organism (Kulp & Kuehn, 2010). Among the OMV proteins, antibiotic resistance and nutrient acquisition-related outer membrane proteins critical for survival through roles such as decreasing porin production to reduce the pathogen's susceptibility to antibiotics and increased membrane integrity were also represented (Hutter *et al.*, 2014, Zhang *et al.*, 2015, Grinter *et al.*, 2019, Roszkowiak *et al.*, 2019, Piotrowska *et al.*, 2020).

OMV functions currently include pathogenesis, inter- or intraspecies communication, and survival reviewed in (Jan, 2017, Woith *et al.*, 2019). Phytopathogen OMV-associated roles include biofilm formation, modulation of plant immunity, and virulence; hence, they are predicted to be intrinsic to their biology, as reviewed by (Katsir & Bahar, 2017). For example, *Xanthomonas campestris*, *X. fastidiosa*, and *Pseudomonas syringae* pv. tomato T1 are among GNB that release OMVs that contain various virulence factors (Chowdhury & Jagannadham, 2013, Ionescu *et al.*, 2014, Solé *et al.*, 2015, Nascimento *et al.*, 2016). Therefore, we expected that OMVs exhibit significance in the fitness of *Pbr1692* within microbial communities, its host adaptation, and particularly in virulence as in the other *Pectobacterium* spp. (Piotrowska *et al.*, 2020). The protection conferred by vesicles ensures the long-distance delivery of proteins shielded from proteases in the environment (Orench-Rivera & Kuehn, 2016). In this study, several PCWDEs and proteases (Pel, Peh, Cel, Prt) were identified virulence factors in OMVs. Moreover, the presence of PCWDEs in OMVs coincided with the abundant oligogalacturonide specific porin (KdgM). KdgM is required to uptake the cell wall degradation products used as a carbon source for bacterial growth after its maceration by PCWDEs (Hutter *et al.*, 2014). We validated this finding by showing that OMV cargo macerates potato tuber tissue of a high moisture and low starch content susceptible *Solanum tuberosum* L. cv Mondial three days post-inoculation. The degree of maceration by OMVs compared with *Pbr1692* cells and the cell-free supernatant emphasised that OMVs contribute as a secretion system.

The OMV proteome profile generated in this study also shows that OMVs carry highly conserved pathogen associated molecular patterns (PAMPS) known to interact with plant recognition receptors (PRR) to alert the plant's innate immune system of an attack (Bahar *et al.*, 2016). In this regard, two flagella proteins were identified in the

proteome of *Pbr1692* OMVs (Bahar *et al.*, 2016). Another PAMP found in the OMV proteome was the elongation factor Tu (EF-Tu). These factors could have contributed to *Pbr1692* OMVs' ability to elicit an immune response in *N. benthamiana*. Other studies characterizing OMV functional roles in phytopathogenesis have also discovered PAMPs such as EF-Tu and polysaccharide A, indicating that vesicles have a conserved mechanism for delivering immunomodulatory molecules from the pathogen to the host (McMillan *et al.*, 2021). In addition, an avirulence protein whose canonical secretion system is the T2SS was also implicated in inducing HR. *Pbr1692* AvrL (A0A0M2F4E7) is a homolog of the virulence protein SvX in *Pectobacterium atrosepticum* and an ortholog of AvrM in *D. dadantii*, and both were previously reported to be upregulated *in planta* (Kazemi-Pour *et al.*, 2004, Corbett *et al.*, 2005).

Previously, we showed that *Pbr1692* has the ability to inhibit the growth of *Dickeya* spp. and other bacteria *in vitro* or during the infection of its host (Shyntum *et al.*, 2019). *Pbr1692* produces phospholipases and other antimicrobial substances, including bacteriocins and carbapenem, to kill competitive bacteria. It is possible that this killing is necessitated due to the high demand for nutrients, including iron. As both *D. dadantii* and *Pbr* typically share the same niche, the challenge posed by *D. dadantii* to *Pbr1692* is that PCWDE regulation is often coupled with iron acquisition; hence, there is competition for the available iron and other nutrients (Franza & Expert, 2010). For this reason, *Pbr1692* can eliminate its competitors such as *D. dadantii* through the release of toxins. Towards this end, OMVs have a fitness and survival role in these complex microbial communities (Guerrero-Mandujano *et al.*, 2017). As such, our proteomics data reveal that OMVs produced by *Pbr1692* contain the CdiA effector (A0A0M2EZM6) that causes contact-dependent growth inhibition (CDI). CdiA toxins bind to specific receptors on target bacteria to deliver C-terminal toxin domains to suppress target cell growth (Aoki *et al.*, 2010). In addition, an antibacterial T6SS substrate, Phospholipase A1 (A0A0M2F4N0), was identified with a probability score of 0.886 among the 72 identified OMV and type 6 secreted effector proteins in this study. Predictably, we showed that OMVs isolated from the *Pbr1692* culture had antiproliferative effects on *D. dadantii*. OMVs reduced the *D. dadantii* cells by approximately 95%.

Amid the host-pathogen arms race, antibiotics, hot water, UV light, bacteriophages, and steam treatments are among strategies that have been explored to control *Pectobacterium* spp. (Charkowski, 2015). OMVs deflect bacteriophage attention and mediate antibiotic resistance (Chattopadhyay & Jagannadham, 2015, Li *et al.*, 2016). We predicted that *Pbr1692* OMV cargo OmpA, OmpK, TolC, and LptD facilitate resistance to carbapenem, an antibiotic that *Pbr1692* itself produces and charges at target competitor bacteria via the T6SS (Shyntum *et al.*, 2019)

Conclusions

We demonstrated that *Pbr1692* formed double- and single-membrane OMVs and defined their roles in infection and survival. OMVs appear to be enhancers of *Pbr1692*'s secretion systems, as shown by their diversified cargo dedicated to bacterial virulence and overall fitness in their ecological niche. The diversity also raises concerns regarding the strictness of this secretion system's cargo selection based on its leader sequence-independent secretion. This study has provided additional knowledge about the importance of specific proteins, including some CAZymes, antibiotic agents, and T6SEs selected for non-classical secretion in addition to classical secretion by *Pbr1692*. This work also serves as a springboard for further investigation of OMV crosstalk with the other secretion systems of GNB, such as the T6SS, in the dedicated delivery of anti-plant host effectors.

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Figures and Tables

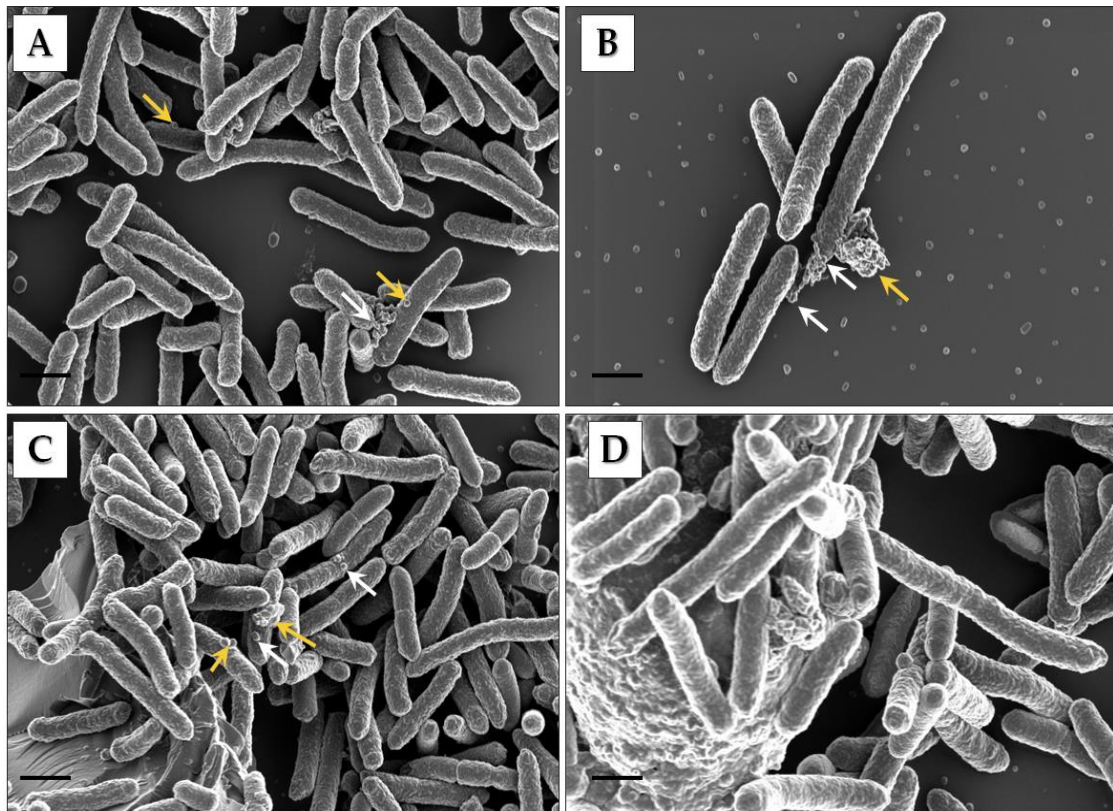


Figure 2.1: Identification of outer membrane vesicles (OMVs) produced by *Pectobacterium brasiliense* 1692. (A, B) In the scanning electron microscope images, gold arrows indicate OMVs of cells and OMV aggregates in the exponential growth phase. The white arrows show vesiculation “hot spots”. (C) The image of cells in stationary growth shows OMVs at the surface of the bacteria indicated by white arrows. Gold arrows indicate OMV aggregates and “hot spots.” (D) The image shows OMVs embedded in the biofilm matrix-like backbone. Scale bars = 1 μm .

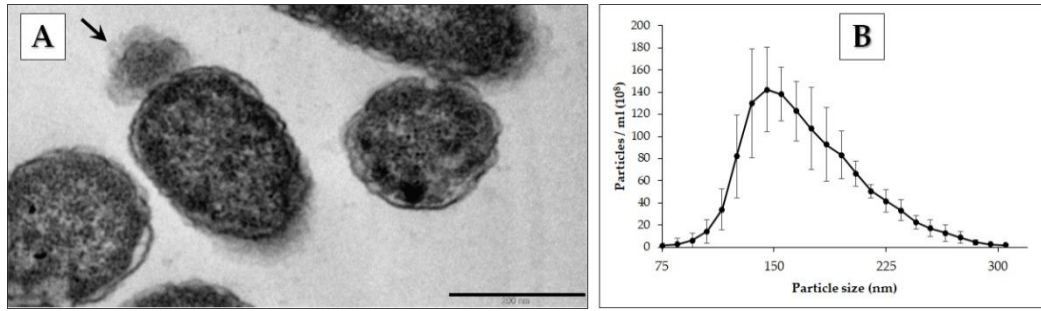


Figure 2.2: Transmission electron microscope identification of budding vesicles on *Pectobacterium brasiliense* 1692 cells and isolated outer membrane vesicles (OMVs) size and distribution. **(A)** The arrow indicates an OMV budding from a *Pbr*1692 cell. Scale bar = 200 nm. **(B)** Nanoparticle tracking analysis size distribution and quantification of isolated OMV assessment shows the distribution of *Pbr*1692 OMVs with an average size of 173.7 ± 1.9 nm isolated in the stationary growth phase.

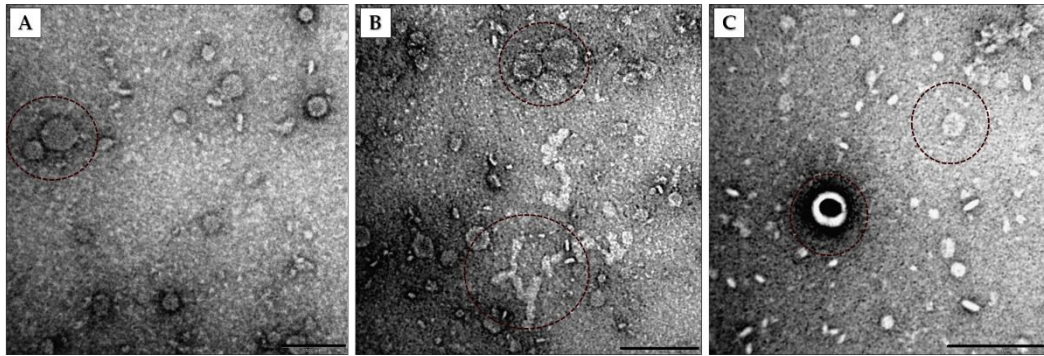


Figure 2.3: Transmission electron microscopy (TEM) of outer membrane vesicles (OMVs) isolated from *Pectobacterium brasiliense* 1692 in the stationary growth phase. (A) The TEM image shows OMVs of different sizes. (B) The image shows OMV aggregates (top) and the OMV chain-like morphology (bottom). (C) The dotted circles indicate a single-membrane vesicle and a double-membrane O-IMV vesicle with a halo around. Scale bars = 200 nm.

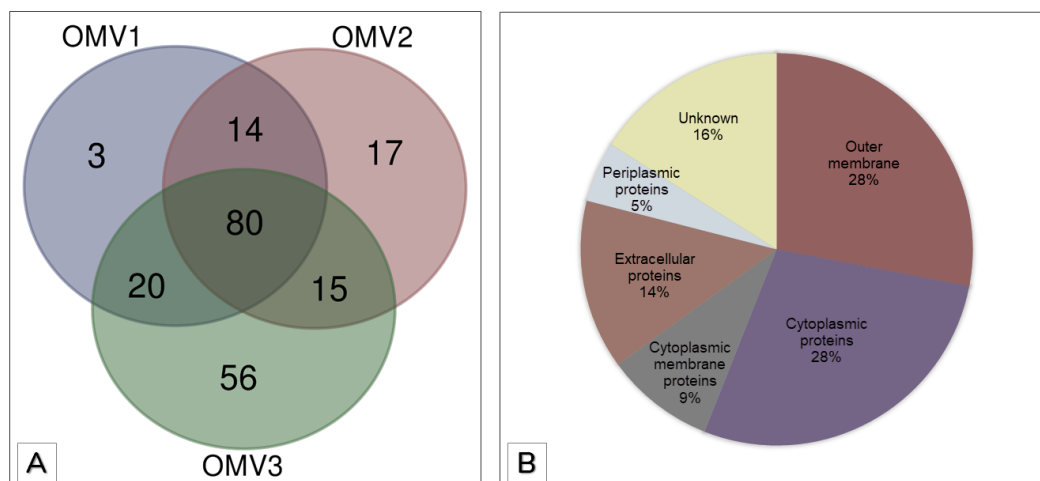


Figure 2.4: Shared and exclusive outer membrane vesicle (OMV) associated proteins of biological replicates and shared protein localisation predictions. **(A)** The Venn diagram indicates shared and exclusive proteins of the three biological replicate OMVs isolated from *Pectobacterium brasiliense* 1692 cultures. Eighty proteins were found present in all three biological replicates. **(B)** The pie chart shows the percentages of shared OMV proteins' predicted localisations.

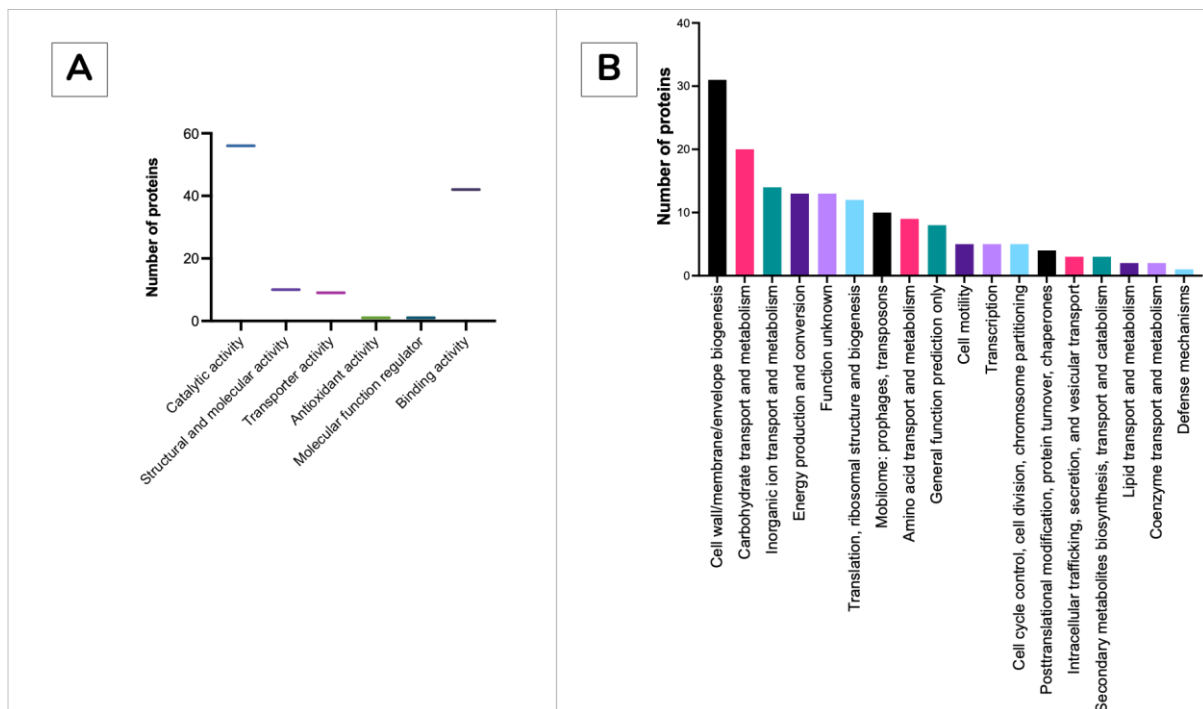


Figure 2.5: Outer membrane vesicle protein molecular functions and protein clusters of orthologous groups classification. **(A)**. The graph shows the molecular ontology entries against the number of associated proteins. **(B)** The chart shows protein functional group categories and the corresponding number of proteins associated with each cluster.

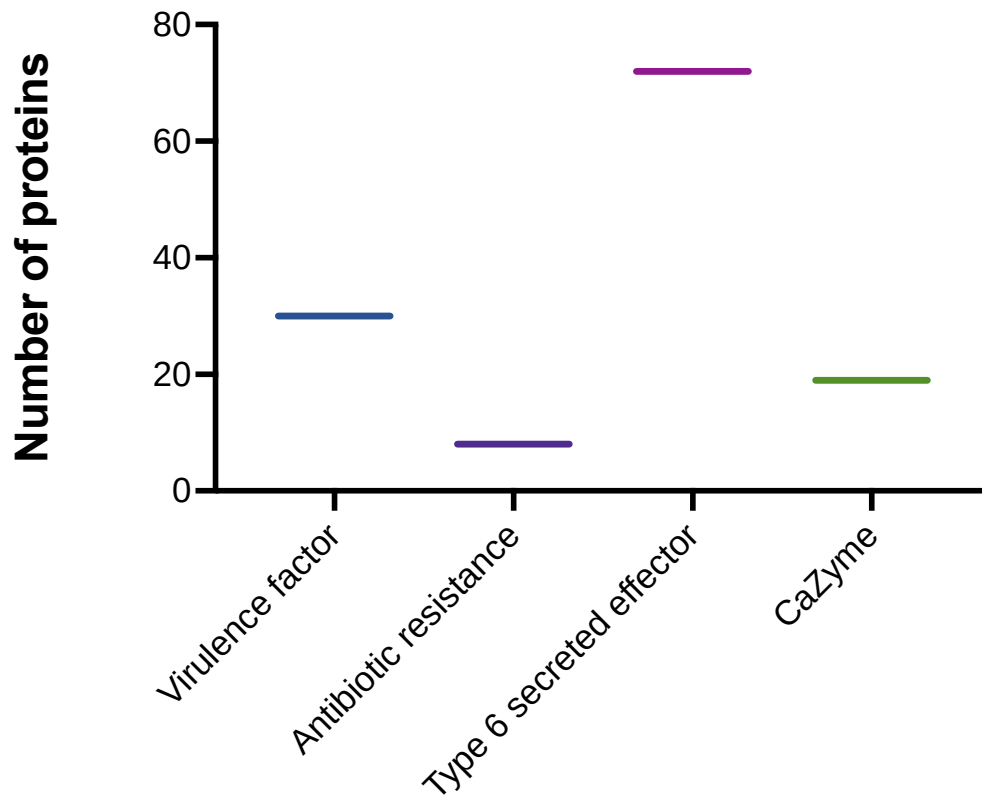


Figure 2.6: Outer membrane vesicle (OMV) proteins associated with virulence, carbohydrate metabolism, stress resistance, and competition. The graph shows the number of OMV virulence factors, carbohydrate-active enzymes, antibiotic resistance agents, and type 6 secreted effectors.

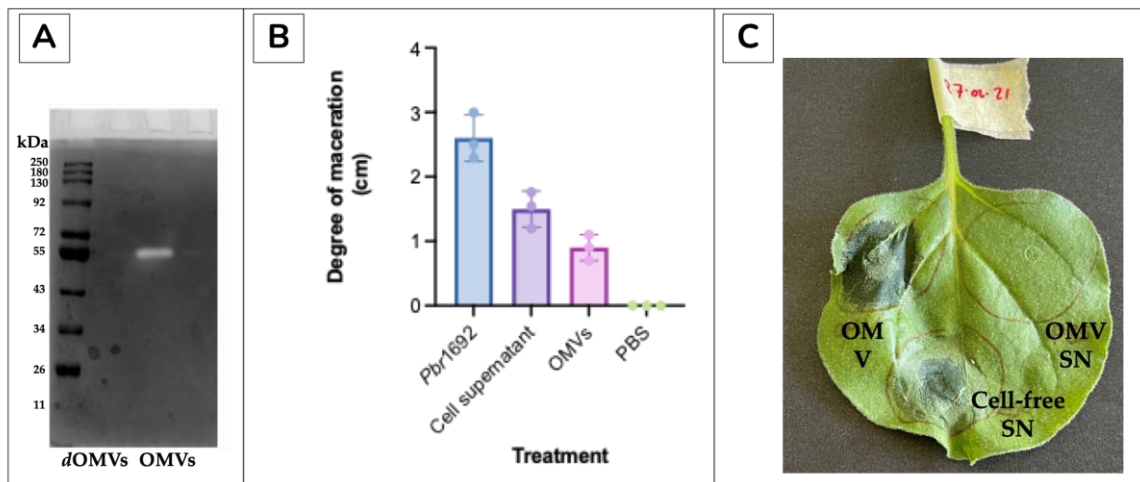


Figure 2.7: Virulence activities of *Pectobacterium brasiliense* 1692 outer membrane vesicles (OMVs). **(A)** OMV protease activity. A 12% sodium dodecyl-sulphate polyacrylamide gel electrophoresis zymogram gel shows *Pbr1692* OMV digestion of gelatine via protease activity. Gelatinase digestion by the OMVs is demonstrated by the white band clearing zone corresponding to a ~55 kDa protein by visualisation using Coomassie Brilliant blue staining. Denatured OMVs were used as a negative control. M = marker; dOMVs = denatured OMVs. **(B)** Soft rot of potato tubers by *Pbr1692* OMVs. The figure shows OMV-associated maceration of potato tuber tissue three days post-inoculation. The cell-free supernatant was used as the positive control, while PBS was used as a negative control. The results show the means of two independent experiments. Error bars represent the standard deviations from the data. Differences between the effect of the control and OMVs were determined to be statistically significant with $p < 0.01$. **(C)** OMVs elicited a hypersensitive response (HR) in three-week-old seedlings of *Nicotiana benthamiana* 24 hpi. The OMV wash step supernatant (SN) and cell-free SN were used as controls.

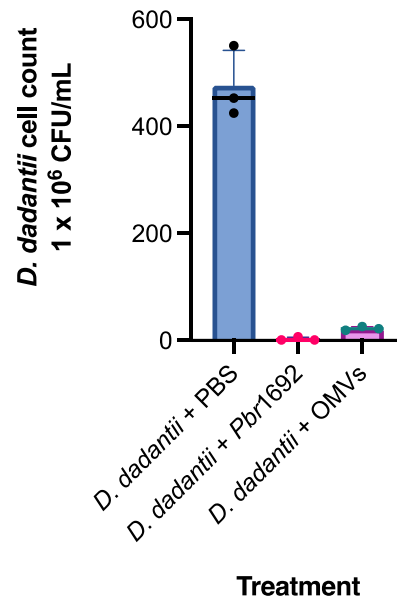


Figure 2.8: Antimicrobial activity of outer membrane vesicles (OMVs). The graph shows *Dickeya dadantii* cells cocultured with phosphate buffered saline (PBS) as a negative control and its growth inhibition in the presence of *Pectobacterium brasiliense* 1692 OMVs and *Pbr1692* cells. The results show the means of two independent experiments. Error bars represent the standard deviations from the data. Differences between the effect of the PBS-negative control with *Pbr1692* cells and OMVs on *D. dadantii* were determined to be statistically significant with $p < 0.01$ in the analysis of variance.

Table 2.1. Mass spectrometry and Mascot comparison data of *Pectobacterium brasiliense* 1692 outer membrane vesicles.

Sample	Total Spectra	Identified Spectra	Identified Peptides	Identified Proteins
OMV1	40,254	1294	629	241
OMV2	39,260	1474	685	262
OMV3	39,726	2167	1002	364
Total	119,240	4935	2316	451
OMV1-3 – outer membrane vesicle				

Table 2.2. The top 50 most abundant outer membrane vesicle proteins of *Pectobacterium brasiliense* 1692 based on iBAQ.

Rank	Protein ID	Description	Molecular Weight [kDa]	Protein Length [aa]	Unique Peptide Number	Gene Ontology IDs	Final Localisation	iBAQ
1	tr A0A6I6X5L4 A0A6I6X5L4_9GAMM	Uncharacterised protein	52.67	492	7	No information	Unknown	7865.322
2	tr A0A086EQL9 A0A086EQL9_9GAMM	MipA/OmpV family protein	27.47	249	6	GO:0016021	Outer membrane	6915.651
3	tr A0A0M2F0M9 A0A0M2F0M9_9GAMM	Phage tail sheath family protein	51.19	475	11	No information	Unknown	6680.566
4	tr A0A6I6XF41 A0A6I6XF41_9GAMM	Porin	39.82	369	12	GO:0006811 GO:0015288 GO:0009279 GO:0046930	Outer membrane	4079.296
5	tr A0A3S0ZK54 A0A3S0ZK54_9GAMM	Lipoprotein NlpD	35.59	344	7	No information	Outer membrane	2786.763

6	tr A0A0M2F4E7 A0A0M2F4E7_9GAMM	Avirulence protein	68.42	621	15	No information	Unknown	2730.042
7	tr A0A6I6X6T7 A0A6I6X6T7_9GAMM	Serralysin	51.22	476	8	No information	Extracellular	2408.248
8	tr A0A6I6X997 A0A6I6X997_9GAMM	Porin	27.64	243	4	No information	Outer membrane	2390.189
9	tr A0A086ESP1 A0A086ESP1_9GAMM	30S ribosomal protein S4	23.44	206	5	GO:0003735 GO:0019843 GO:0006412 GO:0015935	Cytoplasmic	2368.547
10	tr A0A0M2F5U6 A0A0M2F5U6_9GAMM	Tol-Pal system protein TolB	45.89	430	8	GO:0017038 GO:0042597	Periplasmic	2326,679
11	tr A0A6I6WLG2 A0A6I6WLG2_9GAMM	Endolytic peptidoglycan transglycosylase RlpA	39.08	372	9	GO:0071555 GO:0016829 GO:0008932 GO:0000270 GO:0042834	Extracellular	2241.485

12	tr A0A0M2F1K8 A0A0M2F1K8_9GAMM	Membrane protein	18.30	171	4	GO:0016021 GO:0009279	Outer membrane	2232.414
13	tr A0A0M2F2F9 A0A0M2F2F9_9GAMM	Endoglucanase	54.91	505	5	GO:0005576 GO:0030248 GO:0008810 GO:0030245	Extracellular	2195.773
14	tr A0A3S0ZW77 A0A3S0ZW77_9GAMM	DNA protection during starvation protein	18.47	167	2	GO:0005737 GO:0016722 GO:0006879 GO:0030261 GO:0008199 GO:0006950 GO:0003677	Cytoplasmic	2130.814
15	tr A0A6I6X0X5 A0A6I6X0X5_9GAMM	Porin	27.26	238	6	No information	Outer membrane	2073.776
16	tr A0A6I6WXM4 A0A6I6WXM4_9GAMM	TonB-dependent siderophore receptor	85.60	782	17	GO:0005506 GO:0009279 GO:0004872 GO:0015891	Outer membrane	2064.695

17	tr A0A6I6X4V7 A0A6I6X4V7_9GAMM	Long-chain fatty acid transport protein	46.74	433	7	No information	Outer membrane	1905.703
18	tr A0A0M2F6B4 A0A0M2F6B4_9GAMM	Peptidoglycan-associated protein OS	18.52	170	3	GO:0016021 GO:0009279	Outer membrane	1732.263
19	tr A0A086F0V9 A0A086F0V9_9GAMM	30S ribosomal protein S21	8.49	71	2	GO:0003735 GO:0005840 GO:0016787 GO:0005829 GO:0019843 GO:0000028 GO:0044391 GO:0006412 GO:0022627	Cytoplasmic	1724.587
20	tr A0A0M2F5C1 A0A0M2F5C1_9GAMM	60 kDa chaperonin	57.03	548	12	GO:0005737 GO:0042026 GO:0051082 GO:0005524	Cytoplasmic	1668.284
21	tr A0A6I6WLX4 A0A6I6WLX4_9GAMM	F5/8 type C domain-containing protein	72.11	683	8	No information	Extracellular	1577.233

22	tr A0A0M2F2C0 A0A0M2F2C0_9GAMM	Glycine zipper 2TM domain- containing protein	15.48	155	2	GO:0019867	Outer membrane	1402.157
23	tr A0A6I6X4G9 A0A6I6X4G9_9GAMM	Vitamin B12 transporter BtuB	68.71	625	11	GO:0015235 GO:0046872 GO:0046930 GO:0006811 GO:0015288 GO:0004872 GO:0009279	Outer membrane	1394.331
24	tr A0A086EV21 A0A086EV21_9GAMM	Major outer membrane lipoprotein Lpp	8.40	78	2	GO:0009279 GO:0019867	Outer membrane	1370.143
25	tr A0A086EK57 A0A086EK57_9GAMM	Aspartate ammonia- lyase	52.59	479	2	GO:0006099 GO:0006531 GO:0008797	Cytoplasmic	1357.524
26	tr A0A086ESI7 A0A086ESI7_9GAMM	Elongation factor Tu	43.22	394	8	GO:0005737 GO:0003746 GO:0005622 GO:0003924 GO:0005525	Cytoplasmic	1357.145

27	tr A0A6I6X1L4 A0A6I6X1L4_9GAMM	Arabinogalactan endo-beta-1,4-galactanase	56.21	507	4	GO:0015926 GO:0031218 GO:0008152	Cytoplasmic membrane	1346.036
28	tr A0A086EDQ7 A0A086EDQ7_9GAMM	50S ribosomal protein L18 OS	12.71	117	2	GO:0003735 GO:0019843 GO:0005840 GO:0006412	Cytoplasmic	1340.468
29	tr A0A0M2EXV1 A0A0M2EXV1_9GAMM	Baseplate protein	20.34	193	3	No information	Unknown	1321.959
30	tr A0A6I6X2M0 A0A6I6X2M0_9GAMM	Flagellar hook-associated protein 1	59.66	564	5	GO:0044780 GO:0005576 GO:0071973 GO:0009424 GO:0005198	Extracellular	1223.784
31	tr A0A0M2F0F1 A0A0M2F0F1_9GAMM	Membrane protein	23.01	210	4	GO:0016021 GO:0009279	Outer membrane	1198.896
32	tr A0A086EVT8 A0A086EVT8_9GAMM	Membrane protein	19.83	190	3	No information	Unknown	1155.032

33	tr A0A6I6X7Z6 A0A6I6X7Z6_9GAMM	Phage tail protein	71.45	663	8	No information	Unknown	1079.253
34	tr A0A433N765 A0A433N765_9GAMM	Flagellin	30.05	290	7	GO:0005576 GO:0071973 GO:0009420 GO:0005198	Extracellular	1071.265
35	tr A0A0M2F3M0 A0A0M2F3M0_9GAMM	Pectate lyase	40.57	374	5	GO:0030570 GO:0016829 GO:0000272 GO:0005576 GO:0045490 GO:0046872	Extracellular	979.6334
36	tr A0A0M2F635 A0A0M2F635_9GAMM	Membrane protein	50.45	463	7	GO:0005215 GO:0019867	Outer membrane	906.776
37	tr A0A6I6X7V5 A0A6I6X7V5_9GAMM	TonB-dependent receptor	78.19	701	6	GO:0006810 GO:0009279 GO:0004872	Outer membrane	887.6531
38	tr A0A433N6R3 A0A433N6R3_9GAMM	Flagellar hook-associated protein 2	50.76	474	9	GO:0007155 GO:0005576 GO:0009424	Extracellular	871.272

39	tr A0A086EFY4 A0A086EFY4_9GAMM	50S ribosomal protein L17	14.73	130	3	GO:0003735 GO:0005840 GO:0006412	Cytoplasmic	870.2862
40	tr A0A06I6WY80 A0A06I6WY80_9GAMM	Pectate lyase	40.45	375	5	GO:0016829 GO:0005576 GO:0000272	Extracellular	836.9782
41	tr A0A086EHI7 A0A086EHI7_9GAMM	Membrane-bound lytic murein transglycosylase	39.88	357	3	GO:0008933 GO:0016998 GO:0000270 GO:0071555 GO:0009279	Unknown	805.2442
42	tr A0A0M2F7U3 A0A0M2F7U3_9GAMM	Penicillin-binding protein activator LpoA	72.40	672	5	GO:0031241 GO:0030234 GO:0008360 GO:0009252	Unknown	770.3906
43	tr A0A086ESF5 A0A086ESF5_9GAMM	Pectate lyase	40.21	374	5	GO:0030570 GO:0016829 GO:0000272 GO:0005576 GO:0045490 GO:0046872	Extracellular	746.1676

44	tr A0A0M2EW43 A0A0M2EW43_9GAMM	Phosphate-binding protein PstS	36.84	346	5	GO:0043190 GO:0035435 GO:0042301	Unknown	743.0872
45	tr A0A0M2F4N0 A0A0M2F4N0_9GAMM	Phospholipase A1	33.35	290	3	GO:0006629 GO:0008970 GO:0016020 GO:0052739 GO:0102567 GO:0102568 GO:0004623 GO:0052740	Outer membrane	650.1864
46	tr A0A086EA76 A0A086EA76_9GAMM	Ribose-phosphate pyrophosphokinase	34.34	315	3	GO:0005737 GO:0016301 GO:0009156 GO:0000287 GO:0004749 GO:0009165 GO:0009116 GO:0006015 GO:0005524	Cytoplasmic	648.6135
47	tr A0A086EWE3 A0A086EWE3_9GAMM	Outer membrane protein assembly factor BamA	89.09	810	5	GO:0051205 GO:0016021 GO:0009279 GO:0043165	Outer membrane	646.6694

48	tr A0A433N5X5 A0A433N5X5_9GAMM	Putative lipoprotein YajI	20.43	189	2	No information	Cytoplasmic membrane	628.1156
49	tr A0A086E9U3 A0A086E9U3_9GAMM	LPS-assembly lipoprotein LptE	20.37	184	3	GO:0009279 GO:0043165	Outer membrane	617.4362
50	tr A0A3S1FKD4 A0A3S1FKD4_9GAMM	Penicillin-binding protein 1B	92.49	826	4	GO:0009252 GO:0008955 GO:0016021 GO:0008658 GO:0071555 GO:0008360 GO:0046677 GO:0009274 GO:0008233	Cytoplasmic membrane	312.0136

Supplementary Information

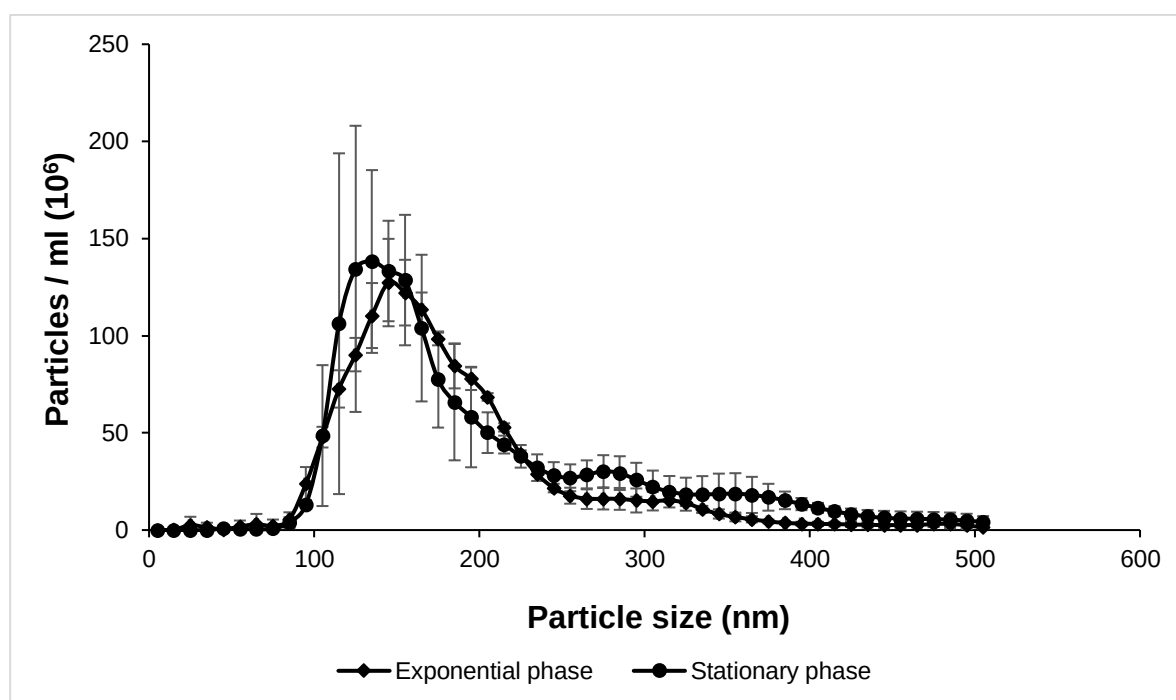


Figure S2.1: Size distribution and concentration of nanoparticles in cell-free supernatants of exponential and stationary phase *Pectobacterium brasiliense* 1692 cultures.

Table S2.1. Proteins identified in three *Pectobacterium brasiliense* 1692 outer membrane vesicle biological replicates.

Protein ID	Protein Mass (kDa)	Localisation
tr A0A086EQL9 A0A086EQL9_9GAMM	27.47	Outer membrane
tr A0A6I6XF41 A0A6I6XF41_9GAMM	39.82	Outer membrane
tr A0A6I6X5L4 A0A6I6X5L4_9GAMM	52.67	Unknown
tr A0A0M2F0M9 A0A0M2F0M9_9GAMM	51.19	Unknown
tr A0A6I6X997 A0A6I6X997_9GAMM	27.64	Outer membrane
tr A0A3S0ZW77 A0A3S0ZW77_9GAMM	18.47	Cytoplasmic
tr A0A3S0ZK54 A0A3S0ZK54_9GAMM	35.59	Outer membrane
tr A0A0M2F5C1 A0A0M2F5C1_9GAMM	57.03	Cytoplasmic
tr A0A0M2F2F9 A0A0M2F2F9_9GAMM	54.91	Extracellular
tr A0A0M2F5U6 A0A0M2F5U6_9GAMM	45.89	Periplasmic
tr A0A0M2F0F1 A0A0M2F0F1_9GAMM	23.01	Outer membrane
tr A0A086ESP1 A0A086ESP1_9GAMM	23.44	Cytoplasmic
tr A0A086F0V9 A0A086F0V9_9GAMM	8.49	Cytoplasmic
tr A0A6I6X0X5 A0A6I6X0X5_9GAMM	27.26	Outer membrane
tr A0A0M2F1K8 A0A0M2F1K8_9GAMM	18.30	Outer membrane
tr A0A6I6WLG2 A0A6I6WLG2_9GAMM	39.08	Extracellular
tr A0A086EDQ7 A0A086EDQ7_9GAMM	12.71	Cytoplasmic
tr A0A433N765 A0A433N765_9GAMM	30.05	Extracellular
tr A0A6I6WXM4 A0A6I6WXM4_9GAMM	85.60	Outer membrane
tr A0A6I6X4V7 A0A6I6X4V7_9GAMM	46.74	Outer membrane
tr A0A6I6WLX4 A0A6I6WLX4_9GAMM	72.11	Extracellular
tr A0A086EV21 A0A086EV21_9GAMM	8.40	Outer membrane
tr A0A0M2F4N0 A0A0M2F4N0_9GAMM	33.35	Outer membrane
tr A0A086ESI7 A0A086ESI7_9GAMM	43.22	Cytoplasmic
tr A0A6I6X1L4 A0A6I6X1L4_9GAMM	56.21	Cytoplasmic membrane
tr A0A0M2F6B4 A0A0M2F6B4_9GAMM	18.52	Outer membrane
tr A0A6I6X2M0 A0A6I6X2M0_9GAMM	59.66	Extracellular
tr A0A3S1FKD4 A0A3S1FKD4_9GAMM	92.49	Cytoplasmic membrane
tr A0A0M2F2C0 A0A0M2F2C0_9GAMM	15.48	Outer membrane
tr A0A086EFY4 A0A086EFY4_9GAMM	14.73	Cytoplasmic
tr A0A6I6X4G9 A0A6I6X4G9_9GAMM	68.71	Outer membrane
tr A0A433N6R3 A0A433N6R3_9GAMM	50.76	Extracellular
tr A0A0M2F635 A0A0M2F635_9GAMM	50.45	Outer membrane
tr A0A0M2F3M0 A0A0M2F3M0_9GAMM	40.57	Extracellular
tr A0A0M2EXV1 A0A0M2EXV1_9GAMM	20.34	Unknown
tr A0A086EVT8 A0A086EVT8_9GAMM	19.83	Unknown
tr A0A6I6X7Z6 A0A6I6X7Z6_9GAMM	71.45	Unknown
tr A0A6I6X5L8 A0A6I6X5L8_9GAMM	29.21	Unknown
tr A0A0M2F7U3 A0A0M2F7U3_9GAMM	72.40	Unknown
tr A0A0M2EW43 A0A0M2EW43_9GAMM	36.84	Unknown
tr A0A6I6WY80 A0A6I6WY80_9GAMM	40.45	Extracellular

tr A0A086EA76 A0A086EA76_9GAMM	34.34	Cytoplasmic
tr A0A086EH17 A0A086EH17_9GAMM	39.88	Unknown
tr A0A086EDP7 A0A086EDP7_9GAMM	26.04	Cytoplasmic
tr A0A086ESF5 A0A086ESF5_9GAMM	40.21	Extracellular
tr A0A086EEV5 A0A086EEV5_9GAMM	42.10	Unknown
tr A0A0M2EWG4 A0A0M2EWG4_9GAMM	39.01	Cytoplasmic
tr A0A086E9U3 A0A086E9U3_9GAMM	20.37	Outer membrane
tr A0A086ESP0 A0A086ESP0_9GAMM	13.85	Cytoplasmic
tr A0A086EZD1 A0A086EZD1_9GAMM	37.37	
tr A0A086EZT0 A0A086EZT0_9GAMM	34.26	Outer membrane
tr A0A433NDC9 A0A433NDC9_9GAMM	99.64	Cytoplasmic
tr A0A6I6WTW7 A0A6I6WTW7_9GAMM	76.41	Cytoplasmic
tr A0A433N5X5 A0A433N5X5_9GAMM	20.43	Cytoplasmic membrane
tr A0A6I6WMR6 A0A6I6WMR6_9GAMM	42.69	Extracellular
tr A0A086EPF1 A0A086EPF1_9GAMM	14.79	Cytoplasmic
tr A0A6I6X1Y3 A0A6I6X1Y3_9GAMM	60.25	Unknown
tr A0A6I6WLX1 A0A6I6WLX1_9GAMM	71.19	Extracellular
tr A0A0M2F7T5 A0A0M2F7T5_9GAMM	56.43	Outer membrane
tr A0A433N384 A0A433N384_9GAMM	28.03	Cytoplasmic membrane
tr A0A6I6X7V5 A0A6I6X7V5_9GAMM	78.19	Outer membrane
tr A0A0M2EW41 A0A0M2EW41_9GAMM	24.00	Unknown
tr A0A6I6X1C9 A0A6I6X1C9_9GAMM	97.46	Outer membrane
tr A0A3S0YB11 A0A3S0YB11_9GAMM	28.03	Unknown
tr A0A6I6X0P1 A0A6I6X0P1_9GAMM	87.15	Outer membrane
tr A0A086F0E3 A0A086F0E3_9GAMM	71.85	Unknown
tr A0A3S0Y0I9 A0A3S0Y0I9_9GAMM	40.67	Cytoplasmic
tr A0A3S0ZUC9 A0A3S0ZUC9_9GAMM	44.52	Cytoplasmic membrane
tr A0A0M2EY17 A0A0M2EY17_9GAMM	29.63	Unknown
tr A0A0M2F469 A0A0M2F469_9GAMM	30.04	Cytoplasmic
tr A0A086E8F3 A0A086E8F3_9GAMM	61.14	Cytoplasmic
tr A0A6I6X605 A0A6I6X605_9GAMM	76.81	Outer membrane
tr A0A3S1AE13 A0A3S1AE13_9GAMM	71.55	Extracellular
tr A0A086EP44 A0A086EP44_9GAMM	52.70	Cytoplasmic
tr A0A2I2MPT4 A0A2I2MPT4_9GAMM	32.52	Cytoplasmic
tr A0A6I6X486 A0A6I6X486_9GAMM	89.52	Outer membrane
tr A0A6I6X1W7 A0A6I6X1W7_9GAMM	32.41	Unknown
tr A0A6I6XAU5 A0A6I6XAU5_9GAMM	56.40	Periplasmic
tr A0A086EWE3 A0A086EWE3_9GAMM	89.09	
tr A0A3S1FJP1 A0A3S1FJP1_9GAMM	45.71	Unknown
tr A0A086ELK2 A0A086ELK2_9GAMM	35.29	Cytoplasmic
tr A0A6I6WS59 A0A6I6WS59_9GAMM	55.88	Outer membrane
tr A0A086EIK2 A0A086EIK2_9GAMM	42.57	
tr A0A6I6WX09 A0A6I6WX09_9GAMM	71.67	Extracellular
tr A0A086EYR9 A0A086EYR9_9GAMM	58.04	Cytoplasmic membrane
tr A0A433ND77 A0A433ND77_9GAMM	50.46	Unknown
tr A0A6I6X0K1 A0A6I6X0K1_9GAMM	50.21	Cytoplasmic

tr A0A6I6X7E6 A0A6I6X7E6_9GAMM	84.82	Cytoplasmic
tr A0A086EWH7 A0A086EWH7_9GAMM	41.74	Outer membrane
tr A0A086EG61 A0A086EG61_9GAMM	45.20	Cytoplasmic
tr A0A086ELC2 A0A086ELC2_9GAMM	55.27	Cytoplasmic
tr A0A6I6XC67 A0A6I6XC67_9GAMM	33.60	Extracellular
tr A0A6I6WUB3 A0A6I6WUB3_9GAMM	304.64	Unknown
tr A0A086EK57 A0A086EK57_9GAMM	52.59	Cytoplasmic
tr A0A086ERR7 A0A086ERR7_9GAMM	35.47	Cytoplasmic
tr A0A6I6WUC6 A0A6I6WUC6_9GAMM	73.11	Outer membrane
tr A0A0M2EZG8 A0A0M2EZG8_9GAMM	65.82	Cytoplasmic
tr A0A0M2F5R5 A0A0M2F5R5_9GAMM	72.59	Extracellular
tr A0A3S0ZY27 A0A3S0ZY27_9GAMM	55.65	Cytoplasmic
tr A0A3S0YBY4 A0A3S0YBY4_9GAMM	46.98	Extracellular
tr A0A6I6WT32 A0A6I6WT32_9GAMM	75.09	Outer membrane
tr A0A6I6WNC8 A0A6I6WNC8_9GAMM	82.58	Outer membrane
tr A0A6I6XAN3 A0A6I6XAN3_9GAMM	86.26	Cytoplasmic membrane
tr A0A6I6WZS7 A0A6I6WZS7_9GAMM	66.76	Cytoplasmic membrane
tr A0A6I6WZW3 A0A6I6WZW3_9GAMM	79.50	Outer membrane
tr A0A6I6WX15 A0A6I6WX15_9GAMM	112.79	Periplasmic
tr A0A0M2F3J1 A0A0M2F3J1_9GAMM	64.32	Extracellular
tr A0A086ED13 A0A086ED13_9GAMM	66.03	Cytoplasmic membrane
tr A0A3S1FJ38 A0A3S1FJ38_9GAMM	39.99	Cytoplasmic membrane
tr A0A086ELJ0 A0A086ELJ0_9GAMM	71.65	Periplasmic
tr A0A6I6X3W6 A0A6I6X3W6_9GAMM	56.20	Periplasmic
tr A0A3S0ZS14 A0A3S0ZS14_9GAMM	96.44	Cytoplasmic
tr A0A6I6X4B6 A0A6I6X4B6_9GAMM	31.13	Cytoplasmic membrane
tr A0A0M2F7F0 A0A0M2F7F0_9GAMM	48.60	Cytoplasmic membrane
tr A0A3S0XI74 A0A3S0XI74_9GAMM	77.44	Cytoplasmic
tr A0A086EYY7 A0A086EYY7_9GAMM	51.73	Cytoplasmic
tr A0A0M2F307 A0A0M2F307_9GAMM	57.66	Periplasmic
tr A0A086EP60 A0A086EP60_9GAMM	55.00	Cytoplasmic
tr A0A0M2EZ70 A0A0M2EZ70_9GAMM	76.54	
tr A0A6I6X625 A0A6I6X625_9GAMM	93.64	Cytoplasmic
tr A0A433MZD1 A0A433MZD1_9GAMM	155.18	Cytoplasmic
tr A0A0M2EZM6 A0A0M2EZM6_9GAMM	270.08	Outer membrane
tr A0A433N166 A0A433N166_9GAMM	78.16	Outer membrane
tr A0A3S0Y9B8 A0A3S0Y9B8_9GAMM	109.89	Unknown
tr A0A6I6WUJ6 A0A6I6WUJ6_9GAMM	53.29	
tr A0A0M2F4E7 A0A0M2F4E7_9GAMM	68.42	Unknown
tr A0A6I6X6T7 A0A6I6X6T7_9GAMM	51.22	Extracellular
tr A0A3S0YAW4 A0A3S0YAW4_9GAMM	48.69	Outer membrane
tr A0A086EYS9 A0A086EYS9_9GAMM	48.14	Cytoplasmic

CHAPTER THREE – Secretome analysis of the type 6 secretion system of *Pectobacterium brasiliense* 1692

Abstract

The type 6 secretion system (T6SS) of Gram-negative bacteria (GNB) is primarily used in contact dependent bacterial competition and injects effectors into the extracellular space and eukaryotic cells. Thus, the T6SS has great implications for bacterial competition, virulence, and survival. For the broad host range potato pathogen, *Pectobacterium brasiliense* 1692 (*Pbr*1692), T6SS-mediated competition is recruited in a tissue-specific manner. However, no other roles have been described. As previously highlighted (Chapter Two), some of the proteins contained in the outer membrane vesicles (OMVs) of *Pbr*1692 were predicted as T6SS substrates, suggesting that these are potentially shared between the T6SS and OMVs. Therefore, based on the annotations of the OMV/T6SS substrates, which also included T2SS secreted substrates, we hypothesised that the T6SS might share substrates with other GNB secretion systems. Indeed, *Pbr*1692 secreted 1479 proteins, 16% of which overlapped between T1SS-T4SS and T6SS according to a comprehensive *Pbr*1692 proteome *in silico* analysis using Bastion tools. Using label-free quantitative (LFQ) mass spectrometry of *Pbr*1692 T6SS-active and T6SS-inactive strains cultured in minimal media supplemented with potato tuber extracts, 446 *Pbr*1692 secreted proteins *in vitro* were identified. Comparison of secretomes of the T6SS-inactive against the T6SS-active background resulted in the identification of 49 T6SS associated proteins. We report 19 and 30 primary substrates and differentially secreted proteins (DSPs) of the T6SS, respectively. The results show that *Pbr*1692 encodes a single T6SS used to secrete proteins of diverse functions. These include a toxin/immunity pair and several predicted virulence proteins, including plant cell wall degrading enzymes (PCWDEs).

Keywords: Secretion systems, type 6 secretion system, secretome analysis, type 6 substrates, differentially secreted proteins

Introduction

Microbes communicate with hosts or other bacteria due to constant selective pressures favouring niche colonisation. During this process, bacteria employ secretory channels whose associated proteins, commonly referred to as effectors, facilitate inter- and intra-species dialogue (Charkowski *et al.*, 2012). Secreted proteins either become substrates that associate with the bacterial outer membrane (OM), are released into the extracellular milieu, or are injected into target cells after secretion (Green & Mecsas, 2016). The effects of effectors manifest in several ways, but their release is frequently associated with (but not limited to) disease development in both animals and plants.

The genus *Pectobacterium* and *Dickeya* make up the Soft Rot *Pectobacteriaceae* (SRP), a family of phytopathogens whose pathogenicity, virulence, and mechanisms for genetic adaptation to the host have received the most attention but have not yet been fully deciphered. Apart from preventative and control measures, disease management is hindered by the lack of translation of research knowledge to practice (Charkowski *et al.*, 2012, Oulghazi *et al.*, 2021). So far, we know that SRP express secretion systems, including outer membrane vesicles (OMVs) and TXSSs (i.e., T1SS-T6SS) that contribute to virulence and survival (Charkowski *et al.*, 2012, Maphosa & Moleleki, 2021). However, not all secretion systems participate in virulence in this family of pathogens as some, like the T1SS and T2SS, have more significant roles as virulence determinants than others, such as T3SS (Holeva *et al.*, 2004, Kim *et al.*, 2011, Charkowski *et al.*, 2012, Hogan *et al.*, 2013). In SRP, the T2SS is the primary virulence determinant owing to a panel of T2SS-secreted plant cell wall degrading enzymes (PCWDEs), which can break down plant cell walls that act as a barrier against bacteria invasion (Pérombelon, 2002).

There is a wealth of information about the roles of Gram-negative bacteria (GNB) secretion systems to which secreted proteins and their biological characterisation have contributed immensely (Pédron *et al.*, 2014, Christie, 2017, Kanonenberg *et al.*, 2018, Wagner *et al.*, 2018, Korotkov & Sandkvist, 2019, Hui *et al.*, 2021). However, the roles of the T6SS in phytopathogen virulence are still not fully uncovered. So far, the roles of some *Pectobacterium* spp. host extracts in inducing and/or upregulating T6SS genes *in vitro*, and/or *in planta* have been highlighted (Liu *et al.*, 2008, Mattinen *et al.*,

2008, Nykyri *et al.*, 2012, Bellieny-Rabelo *et al.*, 2019). In addition, some phytopathogen bacterial T6SS mutant strains display reduced motility, competitive fitness, virulence, and altered metabolic processes (Zhang *et al.*, 2014, Shyntum *et al.*, 2015, Kamber *et al.*, 2017, Shyntum *et al.*, 2019, Singh, 2020, Benavides *et al.*, 2021). However, it is widely agreed that virulence-related phenotypes are most likely secondary consequences of antibacterial T6SS and that only T6SS effectors with direct and clear action against the plant host should be considered. The actual event of T6SS factor injection into plant hosts and how the T6SS confers virulence to *Pectobacterium* remains unclear (Bernal *et al.*, 2018).

A phylogenetic study showed functional diversity within T6SS clades, concluding that the T6SS could not be grouped based on function (Bernal *et al.*, 2018). The study also observed that the T6SS of plant-associated bacteria were identified in all the clades, suggesting that neither is the T6SS evolution dependent on a species' niche or host (Bernal *et al.*, 2018). Therefore, the T6SS functional specialisation instead comes from the effectors it secretes. Others believe that while the roles of a T6SS are defined principally by its diverse effectors, bacteria adapt their T6SS based on host, niche, or survival mechanism (Speare *et al.*, 2018, Coulthurst, 2019, Monjarás Feria & Valvano, 2020). Over time, we have witnessed the heterogeneity of T6SS effector portfolios and functions. Effector functions include reassembling of the T6SS in recipient sister cells using the injected T6SS subunits hemolysin coregulated protein (Hcp) and valine-glycine repeat G (VgrG), adhesion, biofilm formation, fungal killing, ion uptake, stress response, and host colonisation and manipulation (Cianfanelli *et al.*, 2016, Vettiger & Basler, 2016, Gallique *et al.*, 2017, Lin *et al.*, 2017, Wan *et al.*, 2017, Trunk *et al.*, 2018, Chen *et al.*, 2019, Chen *et al.*, 2020).

One major challenge associated with effector identification is limited knowledge about type 6 secreted effector (T6SE) sequence features, and T6SS substrates do not have a model predictable signature or motif, particularly the cargo effectors that bind non-covalently to T6SS components (Hernandez *et al.*, 2020, Hui *et al.*, 2021). Specialised effectors are T6SS component homologues with effector domains covalently fused to them (Hernandez *et al.*, 2020). Sequence analysis of amino acids occupying the first 50 N-terminal and C-terminal positions of T6SEs showed no indication of strong conservation of any sequence motif at either end of the tested proteins (Wang *et al.*,

2018). For this reason, substrate/effector identification is a challenge yet crucial to comprehending the molecular basis of interaction by the T6SS and targets (Zalguizuri *et al.*, 2019).

Since the discovery of the T6SS, several strategies have been used to identify T6SEs (Lien & Lai, 2017). For example, online bioinformatics databases are instrumental for the automated annotation of the T6SS homologs (An *et al.*, 2018). They thus provide a good resource for possible effector discovery (An *et al.*, 2018, Repizo *et al.*, 2019). In addition, new and improved tools such as PyPredT6, Tbooster, Orgissec, Bastion6, and BastionHub enhance T6SS effector predictions (An *et al.*, 2018, Sen *et al.*, 2019, Zalguizuri *et al.*, 2019, Wang *et al.*, 2021). However, because the T6SSs are species-specific, we are yet to encounter new effectors to expand the T6SS versatility. BastionHub encompasses Hidden Markov Model (HMM) and BastionX predictors that enable users to predict potential substrates of secretion systems. However, HMM only retrieves homologs of available known substrates from the query sequences. BastionX can also predict novel substrates (Wang *et al.*, 2021). Complemented with experimental data, the development of the machine learning predictor BastionX provides the research community with a valuable resource to identify distantly related and unrelated TXSS substrates.

In Chapter One, we highlighted the intricacies of crosstalk, specifically highlighting the interdependence among secretion systems. The T6SS was also shown to be part of the dialogues between the secretion systems, including in the recruitment of OMVs and T4SS-mediated activation of T6SS (Ho *et al.*, 2013, Lin *et al.*, 2017, Li *et al.*, 2021). Our most recent work reports putative T6SS substrates among diverse substrates in *Pbr1692* derived vesicles (Maphosa & Moleleki, 2021). In addition, the T2SS plays a significant role in the virulence of *Pectobacterium* spp., but we cannot ignore the possibility that coregulation with the T6SS complements their shared biological functions (Liu *et al.*, 2008, Silverman *et al.*, 2012). Several T6SS of different genera, including *Pectobacterium*, are also expressed when a quorum is reached (Liu *et al.*, 2008, Silverman *et al.*, 2012). *P. carotovorum* and *P. atrosepticum* PCWDE production is also under QS regulon and is induced in the host or extracts of celery, carrot, tomato, and potato (Moleleki *et al.*, 2017, Agyemang *et al.*, 2020). Further, the T1SS and T2SS are two secretion systems that share the responsibility of PCWDE secretion and both

under the QS regulon (Liu *et al.*, 2008). Furthermore, as evidenced by differential protein secretion (significant differences in the amounts of secreted proteins) by secretion mutants compared to wild type strains, 'secondary' substrate secretion reveals that substrate overlap between secretion systems is frequently overlooked during data discussions (Fritsch *et al.*, 2013, Altindis *et al.*, 2015, Condemine & Le Readout, 2022). Therefore, it is important to investigate substrate overlap or crosstalk between the T6SS and virulence determinants in *Pbr1692*.

The present study investigated the possibility of shared substrates among secretion systems (T1SS-T4SS and T6SS). The study was conducted with a particular focus on the T6SS *in silico* predicted substrates and the *in vitro* secretome data of the indispensable T6SS sheath mutant ($\Delta tssBC$) compared to the wild type *Pbr1692* strain, both grown under virulence gene-inducing conditions (minimal media supplemented with potato tuber extracts). This study used computational predictions and mass spectrometry-based label-free quantitative (LFQ) proteomics to identify T6SS associated proteins. Terms 'secretion system substrate' and 'effector' have become interchangeable (Wang *et al.*, 2021). In this study, proteins identified to be associated with the T6SS refer to T6SS substrates or candidate effectors (not yet experimentally validated to target hosts). Comparison of 49 *in vitro* T6SS substrates and the *in silico* T6SS substrate predictions was used to validate 13 predicted T6SS candidates. To assess substrate sharing potential, *in vitro* T6SS substrates and *in silico* predicted T1SS-T4SS substrates were also compared. Twenty-three T6SS substrates appeared to be shared between T6SS and predicted T1SS-T4SS substrates *in vitro*. Twelve were observed to also show sufficient predicted scores for potential T6SS secretion. The results suggest that the role of *Pbr1692* T6SS involves both primary and secondary protein secretion of *Pbr1692* proteins. We propose this based on the T6SS exclusive T6SS substrates and significantly downregulated secreted protein candidates of a diverse functional portfolio by the T6SS-inactive compared to the T6SS-active strains reported in this study.

Materials and methods

In silico prediction of *Pbr1692* secreted proteins and secretion systems responsible for their secretion

The total *Pbr1692* proteome of 4135 was downloaded from UniProt (<https://www.uniprot.org/proteomes/UP000464068> accessed on 15 June 2021). Twenty-eight sequences with less than 50 amino acids or more than 5000 amino acids were filtered out and excluded from the downstream analysis. The remaining 4107 proteins were submitted to the BastionHub for substrate homology prediction using the Hidden Markov Model (HMM) platform (Wang *et al.*, 2021). The 4107 proteins were also redirected to BastionX for more accurate and integrative substrate predictions and classifications of identified secreted proteins into T1, T2, T3, T4, and T6SS substrates using sequence-derived and physicochemical property-based features and experimentally validated substrates to filter the sequences (Wang *et al.*, 2021). In BastionX, a protein was predicted as secreted if its probability score for secretion was ≥ 0.5 and assigned to a specific secretion system if the secretion system's probability score was higher than other systems' probabilities. The 4107 proteins of *Pbr1692* were also submitted to the Bastion6 platform to predict T6SS substrates using various features from T6SEs sequence profiles, evolutionary information, and physicochemical properties (Wang *et al.*, 2018). A probability score ≥ 0.5 was acceptable for each protein association with the T6SS. A Venn diagram was then constructed to identify common substrates between BastionX and Bastion6 predictions. The signal peptides of the substrates were predicted using SignalP 6.0 (<https://services.healthtech.dtu.dk/service.php?SignalP> accessed on 21 March 2022).

The strains and growth conditions

Pectobacterium brasiliense 1692 wild type (T6SS-active) and *Pbr1692* double Δ tssBC mutant strain (T6SS-inactive) (Shyntum *et al.*, 2019) were grown in triplicates at 31 °C in liquid M9 minimal media supplemented with 10% potato extract of *Solanum tuberosum* cv. Mondial prepared according to Mattinen and co-workers (2007) with slight modifications to identify host induced proteins. Minimal media supplemented with potato tuber extracts, hereon virulence inducing media, moderately mimics *in planta* conditions under which *Pbr1692* T6SS and virulence genes are upregulated

(Mattinen *et al.*, 2007, Mattinen *et al.*, 2008, Bellieny-Rabelo *et al.*, 2019). Potato tubers were blended in water and subsequently filtered using miracloth before centrifugation at 10,000 rpm for 20 min to recover the tuber mixture supernatant. The supernatant was clarified by filtering a second time using a 0.22 µm filter. The supernatant was ultra-filtered twice using Amicon® Ultra-15 (MWCO 3kDa) centrifugal units (Merck, Darmstadt, Germany) to eliminate proteins larger than 3 kDa. Finally, the extract flow-through was sterilised using a 0.22 µm syringe filter and utilised immediately to supplement M9 media. *Pbr1692* cells were grown overnight and diluted 1:30 in fresh Luria Bertani (LB) and grown to exponential phase at OD₆₀₀ of 0.6 (Mattinen *et al.*, 2007). The cells were washed three times with virulence inducing media and then inoculated into 1L of the same media to an OD₆₀₀ of 0.1. Then, cultures were grown by shaking at 120 rpm at 31 °C for 15 h until they reached the stationary phase.

Preparation of supernatant from cultures

Supernatants for proteomic analyses were prepared from stationary phase cultures of the T6SS-active and T6SS-inactive strains. The cells were removed by centrifugation at 10,000× *g* for 20 min. Following this, four protease inhibitor complete EDTA-free tablets (26.4 mol/L) were added per 1L supernatant (Sigma-Aldrich, St Louis, MO, USA, (Mattinen *et al.*, 2007). First, the supernatant was filter sterilised through a 0.22 µm membrane to remove residual bacteria. Then, the cell-free supernatant was concentrated to 10 mL using Amicon® Ultra-15 (MWCO 3kDa) centrifugal units (Merck, Darmstadt, Germany). Samples were filtered through a 0.22 µm filter, snap-frozen in liquid nitrogen, and kept at -80 °C or in dry ice until further analysis.

Protein extraction and proteolysis for label-free experiment

Proteins of three biological replicates per strain were extracted and identified according to the BGI Tech Solutions (Hong Kong Co., Ltd) protocol. Cold acetone was added to samples in 50 mL tubes at a ratio of 1:5; then, the protein samples were refrigerated at -20 °C for 30 min. After, the samples were centrifuged at 25,000× *g* at 4 °C for 15 min, and then the supernatant was discarded. The protein was precipitated by air-drying before adding lysis buffer without SDS L3. A grinder at 60 Hz for 2 min was used to promote protein solubilisation, after which the samples were centrifuged

for 15 min at 25,000× *g* at 4 °C to collect the supernatants. Dithiothreitol (DTT) with the final concentration of 10 mM was added to the samples and then the samples were incubated at 37 °C in a water bath for 30 min. After, iodoacetamide (IAM) at a final concentration of 55 mM was added before the samples were placed in a dark room for 45 min. Centrifugation for 15 min at 25000× *g* at 4 °C was used to recover the protein containing supernatants. Quality control was performed by Bradford assay and SDS-PAGE of protein solutions. To digest the samples, trypsin enzyme was added to 100 µg of each sample at a ratio of 1:20 in a 1.5 ml centrifuge tube and vortexed. After, the samples were centrifuged at low speed for 1 min and incubated at 37 °C for 4 h. The digested peptide solutions were then desalted and freeze-dried.

High performance liquid chromatography and mass spectrometry analysis

The dried peptide samples were reconstituted with mobile phase A (2% acetonitrile, 0.1% formic acid) and centrifuged at 20,000× *g* for 10 min. The resulting supernatants were taken for injection and separated using the Thermo UltiMate 3000 UHPLC. Each sample was first enriched in the trap column, desalted, and then channelled into a tandem self-packed C18 column (75µm internal diameter, 3µm column size, 15cm column length). Separation was performed at a flow rate of 300nL/min by the following effective gradient: 0-5 min, 5% mobile phase B (98% ACN, 0.1% FA); 5-45 min, mobile phase B linearly increased from 5% to 25%; 45-50 min, mobile phase B rose from 25% to 35%; 50-52 min, mobile phase B rose from 35% to 80%; 52-54 min, 80% mobile phase B; 54-60 min, 5% mobile phase B. The nanolitre liquid phase separation end was directly connected to the mass spectrometer. Samples were ionised by a nanoESI source and then passed to a tandem mass spectrometer Q-Exactive HF X (Thermo Fisher Scientific, San Jose, CA) for data dependent acquisition (DDA) mode detection. The main parameters were set as follows: ion source voltage was set to 1.9kV, MS1 scanning range was 350-1500 *m/z*; the resolution was set to 60,000; MS2 starting *m/z* was fixed at 100; the resolution was 15,000. The ion screening conditions for MS2 fragmentation were charge 2+ to 6+, and the top 30 parent ions with the peak intensity exceeding 10,000. The ion fragmentation mode was higher energy collisional dissociation (HCD), and the fragment ions were detected in Orbitrap. The dynamic exclusion time was set to 30 s. The automatic gain control (AGC) was set to: MS1 3E6 MS2 1E5.

MS data analysis and protein identification

MaxQuant (<http://www.maxquant.org>) was used for protein identification. The MS data generated from the high-resolution mass spectrometer was input and searched using the MaxQuant version 1.5.3.30 (Max Planck Institutes of Germany) integrated Andromeda engine (Cox & Mann, 2008) against the UniProt_pbra_cyq.fasta database of 20,714 *Pectobacterium brasiliense* sequences. For protein identification, the parameters were selected as follows: trypsin was chosen as the enzyme, peptide mass tolerance was set to 4.5 ppm, fragment mass tolerance at 20 ppm, and minimal peptide length of 7. Carbamidomethylation (C) was the fixed modification. Oxidation (M), acetyl (protein N-term), deamidated (NQ), Gln->pyro-Glu were set as variable modifications. At the spectrum level, filtering was performed with peptide spectrum match level false discovery rate (PSM-level FDR) $\leq 1\%$, and at the protein level, further filtering was performed with protein-level FDR $\leq 1\%$ to obtain significant identification results.

Protein quantification, differential analysis, and functional annotation

MaxQuant was used to extract the peak areas and calculate protein quantity values. The differentially expressed or secreted proteins (DEPs/DSPs) between the comparison groups (T6SS-inactive vs. T6SS-active) were determined based on the protein quantities of the replicates in the R v4.1 using the MSstats package (Choi *et al.*, 2014). Significance testing was performed using Welch's test. Screening was based on the fold change >1.5 and significant difference p -value <0.05 . Spectral counting was used to determine the relative abundance of the identified proteins in the T6SS-inactive biological replicates compared to the T6SS-active replicates (Liu *et al.*, 2004). MaxQuant was also used for functional annotations of the DEPs/DSPs by performing gene ontology (GO), clusters of orthologous groups (COG), and pathway annotations. Candidate virulence proteins were identified using the Virulence Factor DataBase (VFDB) (Altschul *et al.*, 1997, Liu *et al.*, 2019). *Pbr1692* carbohydrate-active enzyme (CAZyme) annotations were performed using the CAZy database (Drula *et al.*, 2022).

Results

Hypothetically shared secreted proteins between T1SS-T4SS and T6SS

To predict the repertoire of proteins secreted by *Pbr1692* to the extracellular environment or into the host, the *Pbr1692* proteome containing 4107 proteins was divided into secreted (1479) and non-secreted (2628) proteins using BastionX. These proteins were respectively predicted with a probability score of ≥ 0.5 and < 0.5 , with the highest probability of each secreted protein determined to be a substrate of T1SS, T2SS, T3SS, T4SS, and T6SS in an all-inclusive approach. First, each protein was assigned a probability score per secretion system. Then, the secreted proteins were allocated to the most likely secretion systems based on the highest probability score, as indicated by the number of predicted substrates per secretion system in Figure 3.1A. The number of proteins predicted to be secreted via T1SS-T4SS and T6SS is shown by the red and blue bars, respectively.

Among the 1479 putatively secreted proteins predicted by BastionX, only five were substrates of secretion systems that are homologous to the available experimentally validated substrates by the fast BastionHub HMM based predictor. The HMM predicted substrates were three T6SS substrates, T6SS tube protein Hcp (A0A6I6X5V0), major exported protein (A0A086EM34) encoded by *hcp*, and Hcp effector (A0A086ET98), one T2SS substrate YjbH domain-containing protein (A0A6I6WZW3), and one T1SS substrate serralyisin (A0A6I6X6T7). Using the more accurate substrate predictor BastionX comprising a suite of algorithms, the results show that *Pbr1692* potentially secretes 1479 proteins: 1041 via T1SS-T4SS and 438 via T6SS.

In Chapter Two, we identified hypothetically shared substrates between OMVs and the T6SS using Bastion6, a machine learning predictor of T6SEs. The OMV/T6SS data extrapolated in Figure 2.6 of Chapter Two includes all 19 predicted T2SS substrates, including PCWDEs (four pectate lyases and one arabinogalactan endo-beta-1,4-galactanase) which were also potential substrates. We, therefore, hypothesised that the T6SS might share substrates with T1SS-T4SS in a hypothetical signature independent manner. To establish if the T6SS has common cargo with other secretion systems, the *Pbr1692* proteome was submitted to Bastion6 for the exclusive

prediction of T6SS substrates based on the prediction tool set probability score of ≥ 0.5 for each T6SS substrate. This cut-off meant that some T1-T4SS substrates predicted by BastionX (Figure 3.1A) formed part of the Bastion6 predicted protein dataset. To identify the potentially shared substrates between the other secretion systems and the T6SS, the Bastion6 and BastionX predicted T6SS substrates were combined and compared to the 1041 BastionX predicted T1-T4SS secreted substrates (Figure 3.1B). Our results show that 231 (22%) of the 1041 T1SS-T4SS predicted substrates are also potentially T6SS substrates (Figure 3.1B). Therefore, there could be substrate overlaps among the secretion systems analysed in this study.

To highlight the potential of sharing substrates and signature independent secretion between the T6SS and the other four systems, the dataset containing the 231 overlapping proteins was used to select proteins with very high probability scores for secretion via the T6SS. We screened the putatively shared output to only include proteins with a cut-off prediction score of ≥ 0.9 , and the list of shared substrates with this cut-off for T6SS secretion is shown in Table 3.1. Based on this, 70 proteins, including PCWDEs, Avirulence L protein, Hcp, VrgG, and phospholipase, were hypothesised to represent T6SS substrates (Table 3.1). Furthermore, 28 proteins (Table 3.1; highlighted in green) were predicted to be exclusively secreted by the T6SS because their probability scores were only above 0.9 for T6SS secretion. Among these were known T6SS substrates such as VgrG and Hcp. To underscore signal sequence-independent secretion potential by the T6SS, the presence or absence of a signal peptide in the 28 proteins was predicted using SignalP 6.0. Thirteen (46%) and 15 (54%) of the proteins had a predictable and non-predictable signal peptide, respectively. The 70 proteins were further evaluated, and it was discovered that 11 of them (highlighted in orange in Table 3.1) had a high chance of secretion by the T2SS, with probability scores lower than for T6SS secretion but still over 0.9 for T2SS secretion (Table 3.1). Thirty-one proteins were predicted as T2SS substrates (blue, Table 3.1) with a high probability of secretion via the T6SS and probability scores less than the probability for T2SS secretion, but over 0.9 for T6SS secretion. Thirty-eight and 19 proteins were identified as candidate virulence factors and carbohydrate-active proteins using the VFDB and CAZy database, respectively (Table 3.1). Overall, our findings reveal that at the high stringency threshold (≥ 0.9 for secretion by a particular system), the T6SS could share cargo with only the T2SS, which is *Pbr1692*'s principal

virulence determinant. This could indicate that the T6SS and T2SS may be able to help each other distribute *Pbr1692* substrates.

Groups of proteins secreted by *Pbr1692* T6SS-active and inactive strains

The secretome of *Pbr1692* was identified from supernatants of T6SS-active and inactive strains grown in virulence inducing media using MS. A total of 4343 peptides and 474 proteins were identified from the MS generated data using the MaxQuant's integrated Andromeda engine. By setting a cut-off of ≥ 2 peptides per protein, 94% (446/474) of the proteins were selected as *Pbr1692* secreted proteins. LFQ using two quantification strategies spectral counting (MS/MS counts) and peptide intensity was employed to screen the proteins. Firstly, MS/MS counts per identified protein were used to identify proteins secreted only by the T6SS-active biological replicates. This semi-quantitative technique utilises the total number of MS/MS spectra identified for a protein to measure its abundance. Secondly, MS/MS counts were used to identify proteins that were only secreted by the T6SS-inactive biological replicates and not by the T6SS-active group. Thirdly, the study used quantitative spectrometric peptide intensity to measure the protein expression in the T6SS-active and inactive groups. Quantitative values obtained for each protein in multiple replicates of the samples were used in Welch's t-test to determine significant differences in the expression and secretion of proteins between the T6SS-inactive and active strains. Fold change >1.5 and the p -value <0.05 was used as the screening criteria for significant differences.

Proteins present only in the T6SS-active strain secretome

The MS/MS counts of the T6SS-inactive replicates were recorded as zero for a protein to be considered as only secreted by the T6SS-active strains (Table 3.2). The presence of proteins in the T6SS-active secretome and not in the inactive T6SS strains showed that these proteins could be primarily T6SS-dependent proteins. Nineteen proteins were identified only in the T6SS-active secretome, among which nine were identified as potential virulence proteins in VFDB, namely serine endoprotease DegQ, four Hcps, two VgrGs, TolC, and flagellar L-ring protein (Table 3.2). Only seven out of the 19 proteins had annotated GO terms. All the 19 proteins identified only in the T6SS-active strain secretome have computational annotations in UniProt with poor annotation scores (experimentally uncharacterised), thus await manual annotation

(Mitchell *et al.*, 2019). In Chapter Two, candidate T6SS substrates were identified in vesicles using primary sequences (Figure 2.6). Only one protein, an F5/8 type C domain-containing protein from the putative candidates was identified as a T6SS-related protein in this chapter.

Proteins present only in the T6SS-inactive strain secretome

For a protein to be considered as only secreted by the T6SS-inactive strains, MS/MS spectral counts of the T6SS-active replicates were recorded as zero (Table S3.1). Twelve proteins were identified only in the T6SS inactive strain secretome and not in the secretome of the T6SS-active strain (Table S3.1). The reason for their secretion only when the T6SS is inactive is unclear. However, it appears T6SS inactivation triggers the expression and secretion of specific proteins not secreted by T6SS-active strains.

Differentially secreted proteins between the T6SS-inactive and T6SS-active strain secretomes

The justification for creating a knockout mutant is that a mutant strain without a certain gene would show phenotypes that are indicative of the missing gene's function. As a result, it is hypothesised that a T6SS-inactive strain lacking the T6SS apparatus will exhibit a phenotype indicating a strain's inability to secrete T6SS-associated substrates (Fritsch *et al.*, 2013). Therefore, comparing the T6SS-inactive and T6SS-active secretome spectral counts allowed us to identify T6SS dependent secretome (Table 3.2). However, in addition to proteins found only in the T6SS-active or inactive secretomes, there were proteins present in both secretomes but differentially expressed and secreted. Peptide intensity quantitation values and statistical analysis allowed for fold change and statistical significance of the differences between groups to be determined (Fritsch *et al.*, 2013). In the current study, the DEPs/DSPs (hereon, DSPs) were proteins whose secretion was significantly low or high (fold change >1.5 and p -value <0.05) in T6SS-inactive group secretomes compared to the T6SS-active group secretomes (Figure 3.2). This implies that the mutation of the T6SS sheath has an unknown impact on the secretion of these proteins. It was not expected that the mutation of the structural components of the T6SS (TssBC sheath) could have effects on other proteins at a transcriptional level. Thus, for the purposes of investigating

BastionX predicted shared secreted substrates among secretion systems, we considered the statistically significantly downregulated proteins in the T6SS-inactive compared to the T6SS-active secretome as T6SS dependent proteins. Although it cannot be ascertained whether these proteins are shared substrates or not, based on the statistical implications, their secretion is affected in the T6SS-inactive strains. As per *in silico* predictions, for the purposes of this study, predicted shared proteins among secretion systems (Figure 3.1B) and proteins identified as DSPs in the T6SS-inactive versus the T6SS-active secretome are referred to as ‘secondary’ substrates of the T6SS.

Forty-nine proteins were differentially secreted (Figure S3.1), with 19 and 30 proteins upregulated and downregulated in the T6SS-inactive versus the T6SS-active strain secretome, respectively (Figure 3.2). Four among the downregulated proteins were previously identified as OMV cargo and predicted as shared substrates with the T6SS (Maphosa & Moleleki, 2021 and Chapter Two, Figure 2.6). Three hundred and forty-one proteins did not show any significant difference in secretion between the strains, as indicated by a lack of statistical difference between the pair groups (Figure 3.2). At this stage, *in vitro* T6SS substrates refers to all the proteins absent in the T6SS-inactive strain secretome yet present in the T6SS-active secretome (Table 3.2) and downregulated proteins in the T6SS-inactive strain secretome against the T6SS-active strain secretome (Figure S3.1). Overall, 49 proteins, including known T6SS substrates, i.e., functional T6SS readout protein Hcp, a major exported protein encoded by *hcp* and VgrG proteins, were identified, thus validating our approach (Table S3.2). Table S3.2 also shows the absence or presence of signal peptides according to SignalP 6.0 which further illuminates signal independent protein secretion by the T6SS *in vitro*.

Functional annotation of differentially secreted proteins

We envisioned that more information on the DSPs would lead to a better understanding of a hypothetical secondary role of the T6SS in the biology of *Pbr1692*. GO and COG annotation analysis were used to assess the functions and functional groups of up- and downregulated proteins in the T6SS-inactive strain relative to the T6SS-active strain, respectively. From this analysis, of the 49 DSPs, 40 were annotated GO terms, with the functional annotations and the corresponding number

of upregulated and downregulated proteins in the T6SS-inactive strain secretome (Figure 3.3A). The exact number of proteins under biological regulation (GO:0065007) and regulation of biological process (GO:0050789) GO terms were upregulated and downregulated in the biological process ontology. More proteins with cellular (GO:0009987) and metabolic process (GO:0008152), as well as cellular component organisation or biogenesis (GO:0071840) functions were downregulated. Three proteins with developmental process (GO:0032502) and two with negative regulation of biological process (GO:0048519) functions were downregulated while no proteins were upregulated in these annotation entries in the T6SS-inactive strain. Three proteins with roles in locomotion were upregulated, and none were downregulated compared to the T6SS-active strain. In the ontology of cellular components, the number of downregulated proteins was also higher than upregulated proteins for almost all the entries except for organelle part (GO:0044422), membrane part (GO:0044425), and extracellular region (GO:0005576) GO terms where equal numbers were observed. Two proteins in the T6SS-inactive strain secretome with extracellular region part (GO:0044421) function were downregulated. No upregulation was observed for this annotation entry. All the annotation entries in the molecular function ontology had more downregulated proteins in the T6SS-inactive strain. Only proteins with antioxidant and regulator activities were downregulated in the T6SS-inactive strain secretome.

We used COGs, VFDB, and CAZy database annotations of DSPs to cement the GO functional assignments (Figure 3.3B). Based on this analysis, 57 proteins assigned to 19 functional categories were predicted to belong to mostly the functional groups involved in translation, ribosomal structure and biogenesis, cell wall or membrane or envelope biogenesis, and intracellular trafficking, secretion, and vesicular transport. Fifteen of the 49 DSPs were predicted as virulence proteins, among which were histidine ABC transporter substrate-binding protein HisJ, phage tail assembly protein, two toxin CdiA, flagellin, and the flagellar basal body rod protein. These were upregulated in the T6SS-inactive group, suggesting a form of compensatory behaviour or loss of negative regulation of the proteins in the absence of the active T6SS. Two superoxide dismutases, serralyisin, flagellar, and peptidyl-prolyl *cis-trans* isomerase, were downregulated putative virulence factors according to VFDB. The T6SS

inactivation also downregulated CAZymes pectinesterase, endoglucanase, and alginate lyase.

Pathway enrichment was used to further define the biological functions of DSPs in the T6SS-inactive versus the T6SS-active secretome. Figure 3.4 shows the distribution of downregulated and upregulated proteins. Downregulation of ribosomal proteins and bacterial secretion proteins VgrG, Hcp, and TolC was observed in the Kyoto encyclopaedia of genes and genomes (KEGG) pathways (data not shown). Two Hcps were predicted to be involved in biofilm formation pathways. TolC was observed as part of the RND efflux pump involved in β lactam resistance, cationic antimicrobial peptide (CAMP) resistance, and plant-pathogen interaction. Serralysin was also associated with (CAMP) resistance. Extracellular solute-binding protein was implicated in iron (III) transport. Endoglucanase was predicted to take part in starch and sucrose metabolism. Upregulated proteins were found in pathways involving carbohydrate and amino acid metabolism.

The findings suggest that downregulated proteins in the T6SS-inactive strain secretome compared to the T6SS-active secretome have diverse biological, cellular component, and molecular functions. The functional diversity alludes to the versatility of the T6SS. After identifying the T6SS substrates secreted *in vitro*, the next step was to compare the 1479 T1SS-T4SS and T6SS (Figure 3.1) secreted substrates predicted *in silico* and the 49 proteins (Table S3.2) identified *in vitro* using LFQ.

Comparison of BastionX predicted secreted proteins and *in vitro* secreted T6SS proteins

A comparison of the proteomics identified T6SS substrates (Table S3.2) and the BastionX predicted substrates of the T1SS, T2SS, T3SS, T4SS, and T6SS (Figure 3.1A) was carried out to establish which BastionX screened proteins were validated *in vitro* as T6SS substrates. Thirteen *in silico* predicted T6SS proteins were confirmed as T6SS substrates *in vitro* (Figure 3.5). These were a superoxide dismutase, three Hcps, two VgrGs, HNH endonuclease, extracellular solute-binding protein, alpha/beta fold hydrolase, alginate lyase, peptidyl-prolyl *cis-trans* isomerase, major exported protein, and formate C-acetyltransferase. The three BastionHub HMM predictor experimentally validated T6SS substrates were among the 13 predicted T6SS

substrates validated *in vitro* in this study. The 13 BastionX predicted T6SS substrates included both proteins identified only in the T6SS-active secretome (7) and downregulated DSP (6). It was observed that 23 of the 49 *in vitro* T6SS substrates were shared between the T6SS and the predicted T1 (3), T2 (7), T3 (11), and T4SS (2) substrates (Figure 3.5). Of these 23, 12 (52%) were predicted at >0.5 probability scores to be potential T6SS substrates (Table 3.3). Among the seven substrates shared between *in vitro* T6SS substrates and BastionX predicted T2SS substrates, six out of seven were predicted with scores between 0.527- 0.937 for T6SS secretion (Table 3.3). These included T2SS candidates pectinesterase and endoglucanase (Table 3.1 and Table 3.3). Overall, the comparison showed that 25 *in silico* deduced T1SS-T6SS secreted proteins were T6SS substrates *in vitro* (Table 3.3). The remaining 13 out of the 49 *in vitro* T6SS substrates were predicted as non-secreted by BastionX. Through these findings we speculate that there is potential substrate overlap *in vitro* based on the differential secretion of some T6SS substrates. The results also show potential substrate overlap based on the *in silico* prediction scores for the *in vitro* T6SS substrates secretion via T1SS-T4SS.

Discussion

The T6SS is an evolutionarily divergent protein delivery system primarily involved in bacterial competition. But, it also participates in activities such as modification and manipulation of eukaryotic cellular processes, stress response, and metal uptake (Ho, 2014, Shyntum *et al.*, 2015, Lin *et al.*, 2017, Si *et al.*, 2017a, Si *et al.*, 2017b, Coulthurst, 2019, Shyntum *et al.*, 2019, Monjarás Feria & Valvano, 2020, Amaya *et al.*, 2022). Likewise, in *Pbr1692*, the T6SS is involved in tissue-specific competition, active in tubers and inactive in the leaves (Shyntum *et al.*, 2019, Miguel, 2020). Antieukaryotic T6SS effector functions have only been extensively defined at a molecular level for animal pathogens (Hachani *et al.*, 2016, Monjarás Feria & Valvano, 2020). For example, several reports on the *Vibrio* actin-crosslinking domain (ACD) have been published (Durand *et al.*, 2012, Heisler *et al.*, 2015, Dutta *et al.*, 2019). However, information on the identified and comprehensively characterised T6SS of plant pathogens remains scarce. The T6SS dependent phenotypes that suggested that the *Pbr1692* T6SS *tssBC* mutation affects virulence, extracellular polysaccharide (EPS) production, and biofilm formation were observed in a study in our laboratory.

These were indicated by reduced potato tissue maceration and low quantities of EPS and biofilm by the T6SS-inactive strain versus the T6SS-active strain (unpublished).

Our published transcriptome data suggested involvement of T6SS in virulence on potato, characterised by upregulation of the T6SS related genes, including four *hcps* and three *vgrGs* alongside most T2SS CAZymes *in planta* (Bellieny-Rabelo *et al.*, 2019). In *P. atrosepticum*, most T6SS genes were upregulated in the asymptomatic and symptomatic stages of systemic plant infection (Liu *et al.*, 2008, Mattinen *et al.*, 2008, Gorshkov *et al.*, 2018). In our previous analysis, the T6SS was shown to potentially share substrates implicated in virulence and survival with OMVs (Maphosa & Moleleki, 2021; Chapter Two of the thesis). Thus, the aim of this study was to identify exclusive and co-secreted substrates of the T6SS and other secretion systems. This way, we anticipated to gain knowledge about the potential roles of the *Pbr1692* T6SS.

Towards this end, we used Bastion6 and BastionX and found that the T6SS might share 231 (16%) of the predicted 1479 secreted cargo with other secretion systems (T1SS, T2SS, T3SS, T4SS). Even though T5SS is one of the six (T1SS-T6SS) secretion systems that are predominantly involved in the secretion of proteins into the extracellular milieu, it is excluded from the BastionX system because T5SS substrate classes either stay bound to the bacterial surface or become freed (Wang *et al.*, 2021). GNB secrete a significant portion (over a third, 33%) of their proteome (Tsirigotaki *et al.*, 2017, Hui *et al.*, 2021). The BastionX predictions that 36% of the *Pbr1692* proteome is secreted were in line with this view.

Type 6 secretion system and T2SS had a higher possibility of 70 common substrates among the secretion systems substrates compared to T1, T3, T4 and T6SS (Table 3.1). As the T6SS system may be species-specific, there is currently no standard for its mechanisms of operation; hence, identifying T6SS substrates or its effector hallmarks has been difficult. In *V. cholerae*, for example, a variety of effector polymorphisms and alleles have been discovered (Unterweger *et al.*, 2014, Kirchberger *et al.*, 2017). In addition, the marker for type six (MIX) and found in type six (FIX) effectors partially identify T6SEs of the *Vibrionaceae* family (Salomon *et al.*, 2014, Ray *et al.*, 2017, Jana *et al.*, 2019). Another example of a species-specific T6SS is found in some *Xanthomonas* spp. whereby the system occupies a role in virulence, while in others, it either does not have any role or plays an indirect one (Zhu *et al.*,

2020, Benavides *et al.*, 2021). In *X. oryzae* pv. *oryzicola* GX01, the two T6SS clusters are not required for virulence in rice (Zhu *et al.*, 2020), but in *X. phaseoli* pv. *manihotis*, the T6SS is involved in virulence (Benavides *et al.*, 2021). There is also an apparent lack of knowledge on the T6SS and T2SS substrates and tool options for their prediction (Cianciotto & White, 2017, Hui *et al.*, 2021, Korotkov & Sandkvist, 2019). BastionX provides the first integrative resource towards identifying T6SS and T2SS substrates via a single platform (Wang *et al.*, 2021).

An additional consideration is that the T3SS, T4SS, and T6SS are found in pathogenicity islands such as phages, plasmids, and transposons (Dobrindt *et al.*, 2015, Morgado & Vicente, 2022). This suggests that versatility for most secretion systems acquired by GNB could be attributed to horizontal gene transfer (HGT) and horizontal movement of effectors (Salomon *et al.*, 2015, Salomon, 2016, Navarro-Garcia *et al.*, 2019, Morgado & Vicente, 2022). More so, we hypothesise that the substrate overlap is for flexibility and efficiency of the newly acquired secretion system. This study has revealed the possible primary and secondary cargo of the T6SS of *Pbr1692* through the various substrates, all of which are likely to entail a wide range of metabolic activity.

To verify the computational findings, *Pbr1692* strains were grown in virulence inducing media to observe the T6SS substrates of *Pbr1692* via a proteomics experimental-based approach. Virulence inducing environmental cues include host chemical signals and host extracts (Mattinen *et al.*, 2008, Borgeaud *et al.*, 2015, Agyemang *et al.*, 2020). In this study, *S. tuberosum* cv. Mondial extract was used to induce the T6SS and virulence genes. Identified through this analysis were 19 and 30 upregulated and downregulated proteins, respectively, in the T6SS-inactive strain secretome compared to the T6SS-active strain secretome. It appears differential secretion by the T6SS has been observed for screening candidates on several occasions, but rarely emphasised (Suarez *et al.*, 2010, Fritsch *et al.*, 2013). For example, a recent report on four new T2SS substrates shows a band of low intensity and thickness for one small protein VirK at the correct size in the T2SS inactive strain supernatant compared to the T2SS active strain supernatant or the complement (Condemine & Le Readout, 2022). VirK is identified as a true substrate of the T2SS, and the reason for the emergence of VirK is unclear. Possible origins could be OMVs, leakage of the protein, or secondary

secretion. VirK and a cellulase were previously found only in the minimal media plus extract T3SS active strain secretome of the phytopathogen *X. citri* subsp. *citri*, as opposed to the T3SS inactive strain secretome (Ferreira *et al.*, 2016). It was concluded that VirK is T3SS dependent (Ferreira *et al.*, 2016).

Recently, a study in *Salmonella* defined the crosstalk between T6SS encoding SPI-19 and T3SS in avian infection (Zhang *et al.*, 2022). The study showed that the T6SS island consisted of regulators of the T3SS. T6SS core component genes were not involved in the regulation observed (Zhang *et al.*, 2022). As the T6SS-inactive strain used in our present study is a structural mutant, it was considered and speculated that there is the possibility of shared secretion responsibility between T6SS and other secretion systems. It was also acknowledged, however, that a structural mutant might suppress other secretion systems' substrates as in the case of *X. citri* subsp. *citri* HrpB4 mutant (Ferreira *et al.*, 2016). In this study, the downregulated proteins were taken to represent putative secondary substrates of the T6SS.

Among these were ribosomal proteins, including 50S ribosomal protein L27. In *V. cholerae* and in *X. citri* subsp. *citri*, 11 and three ribosomal proteins were secreted respectively (Altindis *et al.*, 2015, Ferreira *et al.*, 2016). A study of a *Lactobacillus* sp. reported extra-antimicrobial-ribosomal activity of L27 and L30 of the 50S ribosomal subunit out of seven secreted ribosomal proteins (Pidutti *et al.*, 2018). The study also highlighted other examples of varying additional attributes of ribosomal proteins to protein synthesis (Pidutti *et al.*, 2018). Upon testing, the four ribosomal proteins predicted *in silico* and validated as candidates using proteomics may reveal bacteriocin-like properties of these proteins key in competition as in the above study. In addition to the downregulated proteins, 19 proteins were absent in the T6SS-inactive secretome; hence these were termed exclusive (primary) T6SS substrates. Among these was a F5/8 type C domain-containing protein. Proteins containing this domain were associated with galactose and cell membrane/wall interactions by SRP (Glasner *et al.*, 2008). The number of the primary T6SS substrates identified is double the number of T6SS-related proteins identified in the T6SS secretome of *V. Cholerae* through a comparative proteomics approach (Altindis *et al.*, 2015). LFQ mass spectrometry was used to identify 14 T6SS substrates of *Serratia marcescens* (Fritsch *et al.*, 2013). Almost double the number of proteins (841) identified as secreted

proteins in the above study was identified *in vitro* (446) in the current study (Fritsch *et al.*, 2013). A similar strategy to identify T6SS substrates was adopted where candidates reproducibly detected in at least two of four biological replicates had ≥ 2 peptides with average intensities that were decreased more than four-fold (p -value < 0.05) in the T6SS-inactive strains compared to the T6SS-active strains (Fritsch *et al.*, 2013). T6SS substrates identified in this study included hallmark T6SS proteins, i.e., four Hcps, two VgrGs, and a penicillin binding protein of *Pbr1692* as in *Serratia* (Fritsch *et al.*, 2013). In total, 49 *in vitro* T6SS substrates were identified in this study.

The BastionX tool was capable of successfully predicting 51% of the proteomics identified T6SS substrates. This means that 25 proteins identified by computational sequence analysis as secreted proteins of *Pbr1692* (substrates of T1SS-T6SS) were also identified *in vitro* as T6SS substrates. Twelve of these were indicative of the possible substrate sharing between secretion systems and the diverse qualities and roles of T6SS proteins (Table 3.3). A handful of reports to validate the secretion of some of these proteins by the other secretion systems include endoglucanase and pectinesterase T2SS secretion in soft rots (Toth *et al.*, 2003, Stafford & Hughes, 2007, Coulthurst *et al.*, 2008, Hirano *et al.*, 2009, Pédrón *et al.*, 2014), as well as serralysin, a T1SS substrate (Ravindran *et al.*, 2018). Thirteen experimentally identified T6SS substrates were *in silico* predicted T6SS specific substrates. These were four Hcps, two VgrGs, superoxide dismutase, peptidyl-prolyl cis-trans isomerase, formate C-acetyltransferase, alginate lyase, extracellular solute-binding protein, and an HNH endonuclease.

The experimental results did not confirm majority of the predicted substrates *in silico* or the sharing of the substrates by the T6SS and other systems (Figure 3.1). In fact, there is a deficit in both directions, i.e., *in silico* versus *in vitro*. For example, a quick survey of the number of predicted T3SS secreted substrates casts doubt due to the likely overestimation or exaggeration of T3SS targets. Also, the T3SS is negligible in *Pectobacterium* infection and only one T3SS effector, DspE, is known (Holeva *et al.*, 2004, Kim *et al.*, 2011, Hogan *et al.*, 2013). *In silico* identification bears the limitation of available data for training prediction tools, which may lead to high tailoring of a tool to the given dataset. Prediction requires prior knowledge, yet substrates such as type 3 secreted effectors (T3SEs), type 2 secreted substrates, and T6SEs are largely

diverse, species-specific, or poorly represented in datasets (Wang *et al.*, 2018, Cianciotto & White, 2017, Hui *et al.*, 2021). Phytopathogen T6SEs are a case in point. The BastionX ensemble was trained using 132, 62, 410, 339, and 119 T1SS, T2SS, T3SS, T4SS, and T6SS substrates and 1112 non-substrates, mined exhaustively from literature, respectively (<https://bastionx.erc.monash.edu/download.jsp>) (accessed on 12 July 2022, Wang *et al.*, 2021). DspE and one conserved hypothetical VgrG protein, one conserved hypothetical Hcp protein and two Hcp1 effectors are the only *Pectobacterium* T3SS and T6SS in the BastionHub database, respectively (Wang *et al.*, 2021). Despite this, outputs gathered from the single predictors and the final ensemble with higher accuracy are believed to present avenues of effector discovery not previously considered. In line with new resources, BastionX is the first predictor of T1SS and T2SS secreted proteins. Therefore, we ought to consider assessment of the properties of the predicted TXSS proteins and the ones predicted *in vitro* by this study or a combined large dataset of *Pectobacterium* predicted TXSS substrates to identify new TXSEs for further training of predictors. *Pbr1692* encodes a functional T3SS. Thus, it is possible its effectors are not yet fully identified, as with the case of the T2SS presented in a recent study (Condemine & Le Derout, 2022). In reference to the T2SS, the secretion signal of its substrates after translocation to the periplasm remain an enigma (Desvaux *et al.*, 2009, Korotkov & Sandkvist, 2019). Thus, with improvements, the predictors in BastionX may be quite instrumental in screening for novel candidates.

Proteomics-based investigations, on the other hand, are constrained since not all effectors can be secreted at high quantities *in vitro* and are secreted in response to the surrounding environment, media, or exogenous substances. As in the *V. cholerae* T6SS study, we did not identify any PAAR proteins possibly due to their low expression levels, affecting detection (Altindis *et al.*, 2015). A recent study compared protein expression of *P. carotovorum* subsp. *carotovorum* PccS1 *in vivo* (*in planta*) and *in vitro* on LB media supplemented with plant extract in a two-dimensional gel electrophoresis (2-DE) protein profile comparison (Wang *et al.*, 2018). When compared to *in vitro*, 68 and 65 proteins showed increased and decreased expression *in planta*, respectively. Furthermore, the stability of some proteins necessitates the presence of a chaperone (Lien & Lai, 2017). However, both approaches can be utilised successfully in tandem; while proteomics is based on discovery, sequence analysis is based on knowledge

(Lien & Lai, 2017). In a study on *S. enterica*, differences were observed in the protein profiles of bacteria grown in different growth media (Blair *et al.*, 2013). As a result, we can argue that the conditions chosen for this study may account for the missed predictions. Since plant extracts fail to fully simulate the plant environment and account for the plant response, expression and secretome are likely to vary between growing conditions. Therefore, *in vitro* conditions cannot genuinely represent the setting in the host (Gorshkov *et al.*, 2018, Wang *et al.*, 2018). Likewise, identifying secretion system substrates *in vivo* experimentally would be challenging and time-consuming. Proteins sourced as the *in planta* proteome could also just be a fraction of the proteins expressed during the *in vivo* infection process (Cheng *et al.*, 2010, Wang *et al.*, 2018). As a result of the aforementioned disparities, we expected differences in the *in vitro* and *in silico* results in this study, although both were informative in terms of *Pbr1692* protein secretion. It is important to note, however, that specific modifications (i.e., the proteins were screened based on the tool set positive substrate cut off 0.5 instead of highest probability scores) deliberately applied in this study revealed more than was achievable with the Bastion tools on their own.

So far, we know that the T6SS is primarily a contact-dependent system, although contact-independent killing, T6SS mediated resource uptake, and putative collaboration with OMVs have been reported (Lin *et al.*, 2017, Maphosa & Moleleki, 2021, Song *et al.*, 2021). Therefore, growing *Pbr1692* in a broth may have reduced T6SS mediated secretion *in vitro* (Fritsch *et al.*, 2013). We expected low numbers of antibacterial toxins based on the study's virulence genes-inducing conditions. AHH, WHH, and LHH containing proteins are nucleases found in bacterial polymorphic toxin/immunity systems (Zhang *et al.*, 2011). Bellieny and co-workers (2019) identified transcriptional coregulation between the toxin immunity pair WHH/SMI1_KNR4 families (Pfam entry PF14414 and PF09346) and the T6SS in *Pbr1692*. This study identified the HNH endonuclease to be exclusively secreted via the T6SS (Table 3.2). HNH endonuclease is a WHH nuclease of the HNH/ENDO VII superfamily with conserved WHH (Pfam entry PF14414). We also identified the protein SMI1/KNR4 of Hcp secretion island-2 (HSI-2) only in the T6SS-active secretome (Table 3.2). SMI1/KNR4 (Pfam entry PF09346) is a potential immunity protein involved in contact-dependent inhibition (CDI) systems of *Proteobacteria* (Zhang *et al.*, 2011). Therefore, we concluded that HNH/SMI1_KNR4 is an immunity-toxin pair implicated in contact-

dependent growth inhibition (CDI) by the T6SS. However, evidence to prove HNH secretion has been lacking until now. It is not clear why the immunity protein was also secreted. Through sequence analysis and wet-lab experimental analysis, this study shows that HNH endonuclease is a *Pbr1692* T6SS substrate. Moreover, guilt by association was helpful in the identification of probable T6SS effectors (Bellieny-Rabelo *et al.*, 2019). Confirmation of secretion also shows that transcriptomics indicates the state that precedes protein expression.

Interference competition mediated by the T6SS typically involves peptidoglycan, membrane phospholipids, NAD, and nucleic acids targets (Russell *et al.*, 2012, Russell *et al.*, 2013, Coulthurst, 2019). *Pbr1692*'s fitness against competing bacteria might be due to phage induction, nutrition competition, and antimicrobial compounds (bacteriocins and carbapenem). Bioinformatic assay by Bellieny and co-workers (2019) had identified four potential effectors: a phospholipase, AHH nuclease, WHH nuclease, the Rhs1 effector, and D123 protein found in HSI of *Pbr1692*. However, all of these effectors did not show antibacterial activity against *Escherichia coli* DH5- α during ectopic expression except for AHH nuclease (Bellieny-Rabelo *et al.*, 2019). Competition in bacteria may be passive/exploitative even via the T6SS (Chen *et al.*, 2016, Si *et al.*, 2017a, Si *et al.*, 2017b, Li *et al.*, 2021). The *Pbr1692* T6SS is highly upregulated in potato tubers which strongly suggests host cue or response mediated induction of the T6SS for exploitative competition. Notwithstanding, this system participates in active/interference competition in the host (Shyntum *et al.*, 2019). Our current data only shows one putative toxin and several potential virulence factors under virulence-gene inducing conditions. As mentioned previously, we suspect proteomic analyses may have missed low abundance proteins and those not expressed during sampling (Zalguizuri *et al.*, 2019). The antimicrobial compounds may also have been missed, although similar studies have identified them using the same strategy (Fritsch *et al.*, 2013, Altindis *et al.*, 2015).

TolC was one of the proteins that was only discovered in the T6SS-active secretome and not the T6SS-inactive secretome. Another TolC family protein was downregulated in the T6SS-inactive strain compared to the active strain. TolC is the outer membrane component of the T1SS/RND structure, and a multifunctional protein involved in many critical cellular processes, including antibiotic stress resistance. It is also upregulated

by plant extracts (Marits *et al.*, 1999, Thomas *et al.*, 2014), and in this study, it appears to be T6SS dependent. Indeed, the absence of TolC would affect several cellular processes, including the T1SS assembly, pathogen-host interaction, and resistance. However, a more plausible reason for the presence of TolC in the T6SS-active strain secretome could be that the membrane integrity of T6SS-active strains is at some stage more compromised than that of T6SS-inactive strains under the growth conditions resulting in the loss of TolC from the outer membrane possibly as part of OMVs (Chapter Two). The T1SS of SRP secretes metalloproteases that contribute to virulence (Charkowski *et al.*, 2012). Protease serralyisin, which is a known substrate of the T1SS (Ravindran *et al.*, 2018), was identified in this study. A probability score of 0.994 predicted serralyisin as a T1SS substrate using BastionX. This substrate was also among differentially secreted T6SS substrates *in vitro*. The T2SS and T6SS *in silico* probability scores for this protein were 0.979 and 0.858, respectively. Even with the chance that T1SS assembly was affected, serralyisin was potentially secreted via the T2SS. Curli pore protein CsgG was also differentially secreted by the T6SS-inactive strain compared to the T6SS-active strain (Table S3.2). Curli are surface attached extracellular secreted amyloid-like protein fibres that facilitate bacterial adhesion, biofilm formation, and surface colonisation (Evans & Chapman, 2014, Costa *et al.*, 2015).

Our findings imply that the *Pbr1692* T6SS is involved in the secretion of its core substrate proteins and has an influence on the secretion of other secretion system substrates. The nature of the influence and its biological significance are likely to be complicated, and unclear at this time. The *Pbr1692* HSI-3 island for example, has seven genes, and lacks a *vgrG*. The island appears unrelated to antibacterial functions (Bellieny-Rabelo *et al.*, 2019). Based on our choice of growth media, we anticipated our predictions would define the roles of the T6SS in *Pbr1692* outside of the antibacterial association. As a soft rot pathogen, *Pbr1692* encodes and secretes a high number of PCWDEs during host invasion and infection (Pérombelon, 2002, Charkowski *et al.*, 2012, Hugouvieux-Cotte-Pattat *et al.*, 2014). As mentioned previously, using computational analysis, we discovered that there is a high prediction of T2SS as T6SS potential candidates (likelihood >0.9) in comparison to other secretion systems (T1SS, T3SS, and T4SS). Comparing the *in vitro* T6SS substrates and *in silico* predicted T2SS substrates revealed seven potentially shared virulence

proteins between the two secretion systems. The T6SS/T2SS candidates were a pectinesterase, endoglucanase, phospholipid-binding protein MlaA, flagellar L-ring and FlhE proteins, amidohydrolase family protein, and f5/8 type C domain-containing protein. Pectinesterase is a ubiquitous protein associated with plant cell wall modification and subsequent breakdown. The pectinesterase identified in this study is a homolog of a pectin methylesterase involved in the pectinolytic cascade during host infection (Boraston & Abbott, 2012). Pectin methylesterase A and B are essential for optimal growth of the soft rot phytopathogen *D. dadantii* 3937 on heavily methylated pectic substrates (Boraston & Abbott, 2012). According to our study, endoglucanase is also an extracellular carbohydrate-active enzyme secreted via the *Pbr1692* T2SS and T6SS. Extracellular endoglucanase of *X. citri* subsp. *citri* is essential for full virulence of the phytopathogen, and deletion results in reduced cellulase activity (Xia *et al.*, 2016). *P. brasiliense* SX309 encodes two endoglucanase genes associated with oligosaccharide degradation and conserved in *Pectobacterium* strains (Li *et al.*, 2019). The Flagellar L. ring protein is part of the flagellar T3SS that secretes bacteriocins and controls cell motility and size of *P. carotovorum* subsp. *carotovorum* (Chan *et al.*, 2009, Wu *et al.*, 2021). Noteworthy, flagellar expression, secretion, and motility are affected by T6SS mutations in GNB (de Pace *et al.*, 2011, Liu *et al.*, 2015, Benavides *et al.*, 2021). However, flagellar L ring protein is an outer membrane protein and may have been liberated from the membrane into the media upon the lysis of or shearing from a few *Pbr1692* cells.

Reactive oxygen species (ROS) are one of the main stress factors encountered by *Pbr1692* in the host environment (Mosina & Moleleki, 2018). Superoxide dismutase is an antioxidant under ferric uptake regulator regulation produced by *Pbr1692* to cope with ROS (Tanui *et al.*, 2017). Two superoxide dismutase proteins were identified as T6SS substrates and putative virulence factors via VFDB. The discovery of superoxide dismutase in our dataset is support that the T6SS might be involved in evading host defences (Table S3.2). We also identified an extracellular binding protein involved in iron uptake. This *Pbr1692* T6SS substrate is homologous to the T2SS secreted iron and copper binding protein IbpS of *D. dadantii* 3937 (Gabler *et al.*, 2020). The metal binding protein is potentially involved in protecting *Pbr1692* from ROS via antioxidant activity (Liu *et al.*, 2019). To counter pathogen antioxidant activity, a recent study has proposed copper sulphide nanoparticles (CuS NPs) as low toxicity and

environmentally friendly antibacterial agents that can increase the levels of ROS and lipid peroxidation to destroy the structure and inhibit motility of *Pectobacterium* spp. (Yan *et al.*, 2022).

Deletions of T6SS components in a kiwifruit pathogen *Pseudomonas syringae* pv. *actinidiae* showed diminished competition, biofilm formation, hydrogen peroxide tolerance, and proteolytic activity (Wang *et al.*, 2021). In this study, an inactive T6SS also resulted in the absence and downregulation of Hcp proteins, one which was identified as a protein indirectly implicated in biofilm formation (Figure 3.4). The *Pbr1692* T6SS-inactive strain in the unpublished study exhibited reduced virulence, impaired EPS production and attenuation of biofilm production (unpublished data). EPS is essential for xylem vessel occlusion during *Pbr1692* infection and was shown in *P. atrosepticum* to scavenge ROS and suppress plant responses (Islamov *et al.*, 2021). In another study, a mutant of the T6SS *TssC* gene failed to produce a biofilm or inhibit biofilm formation in another strain. *P. fluorescens* strain MFE01 Hcp1-3 were essential for forming mature biofilm (Gallique *et al.*, 2017). In *Erwinia amylovora*, *P. aeruginosa*, *E. coli*, and melon phytopathogen *Acidovorax citrulli*, the T6SS is also associated with biofilm formation (de Pace *et al.*, 2011, Tian *et al.*, 2015, Tian *et al.*, 2017, Chen *et al.*, 2020). Chen and co-workers reported that the potent biofilm forming isolates showed significantly higher expression levels of T6SS-associated genes than the non-biofilm forming isolates and higher expression levels of *hcp1–3* in the PAO1 biofilm in comparison to the planktonic cells (Chen *et al.*, 2020).

Conclusions

According to the findings of this investigation, *Pbr1692* secretes proteins with unclear biological functions. The descriptions of the identified substrates point to a variety of yet-to-be-demonstrated actions that the T6SS facilitates. Furthermore, it is unknown if the T6SS is directly or indirectly involved in the secretion of the newly identified differentially expressed proteins (pleiotropic). BastionX is a promising platform for screening proteomes, opening up new avenues for effector identification and comparison of secretion system substrates in the future based on the amount of validated *in silico* predictions. It has several restrictions, though. Now that T6SS secreted substrates have been described in this study and its use in bacterial competition in earlier investigations, it is more likely than ever that *Pbr1692* encodes

a single T6SS for both competition and additional unidentified contact independent biological functions. Additional research is necessary to fully comprehend the molecular basis of the T6SS released proteins and substrate overlap among secretion systems in *Pbr1692*.

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Figures and Tables

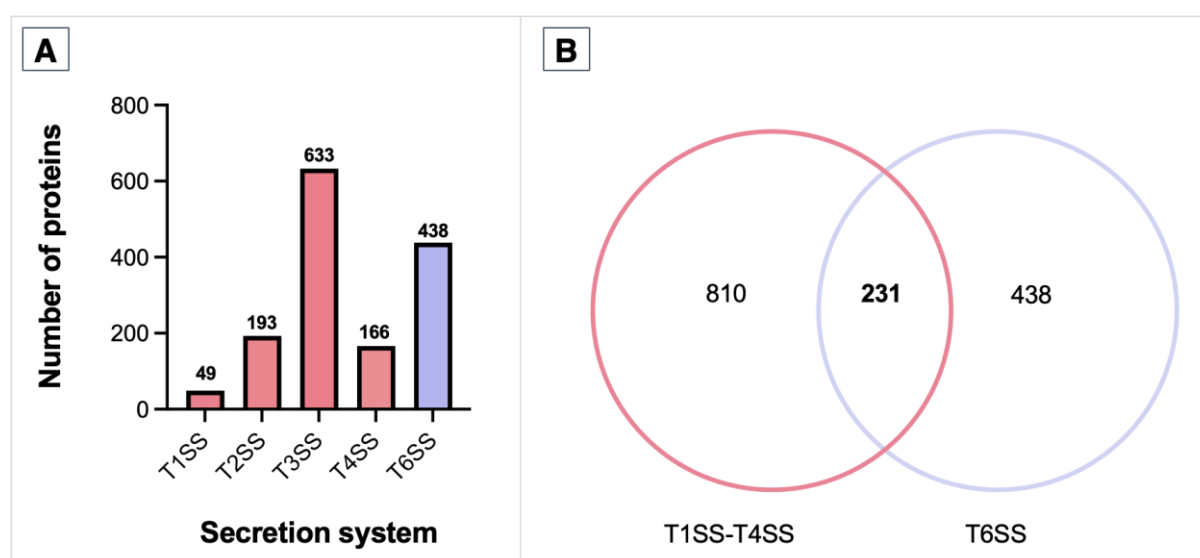


Figure 3.1: Exclusive and shared substrates between the type 6 secretion system (T6SS) of *Pectobacterium brasiliense* 1692 and type 1 secretion system-type 4 secretion system (T1SS-T4SS) based on Bastion6 and BastionX predictions. **(A)** The graph shows secretion systems T1SS-T4SS and T6SS of *Pbr*1692 and the number of proteins predicted to be secreted by each secretion system. **(B)** The Venn diagram shows the shared and exclusive predicted substrates between the T6SS and T1SS-T4SS. Two hundred and thirty-one proteins are potentially secreted via the T6SS and T1SS, T2SS, T3SS, or T4SS at a probability of at least 0.5.

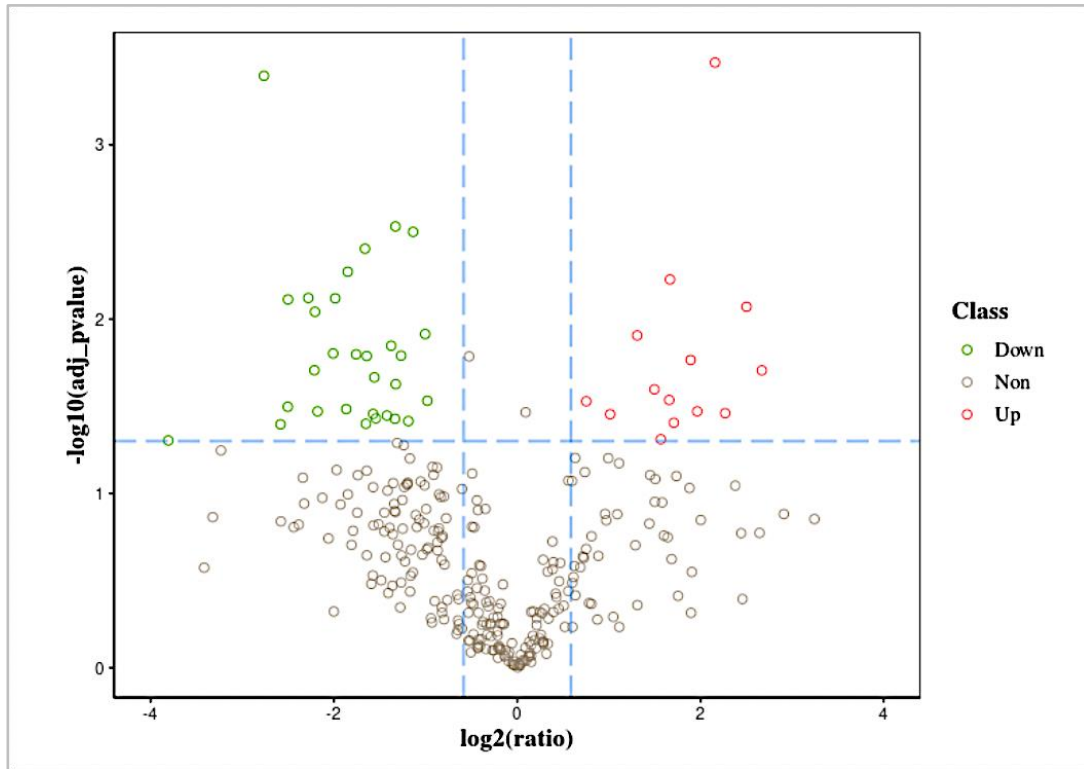


Figure 3.2: Differentially secreted proteins between type 6 secretion system (T6SS) inactive and T6SS-active *Pectobacterium brasiliense* 1692 strains. Each red dot in the volcano plot represents a significantly upregulated protein, the green dot, a significantly downregulated protein, and the grey dot, a non-significantly altered protein. The protein difference multiples are plotted against the corresponding $-\log_{10}$ value ($p < 0.05$) between the *Pbr1692* T6SS-inactive and T6SS-active secreted proteins.

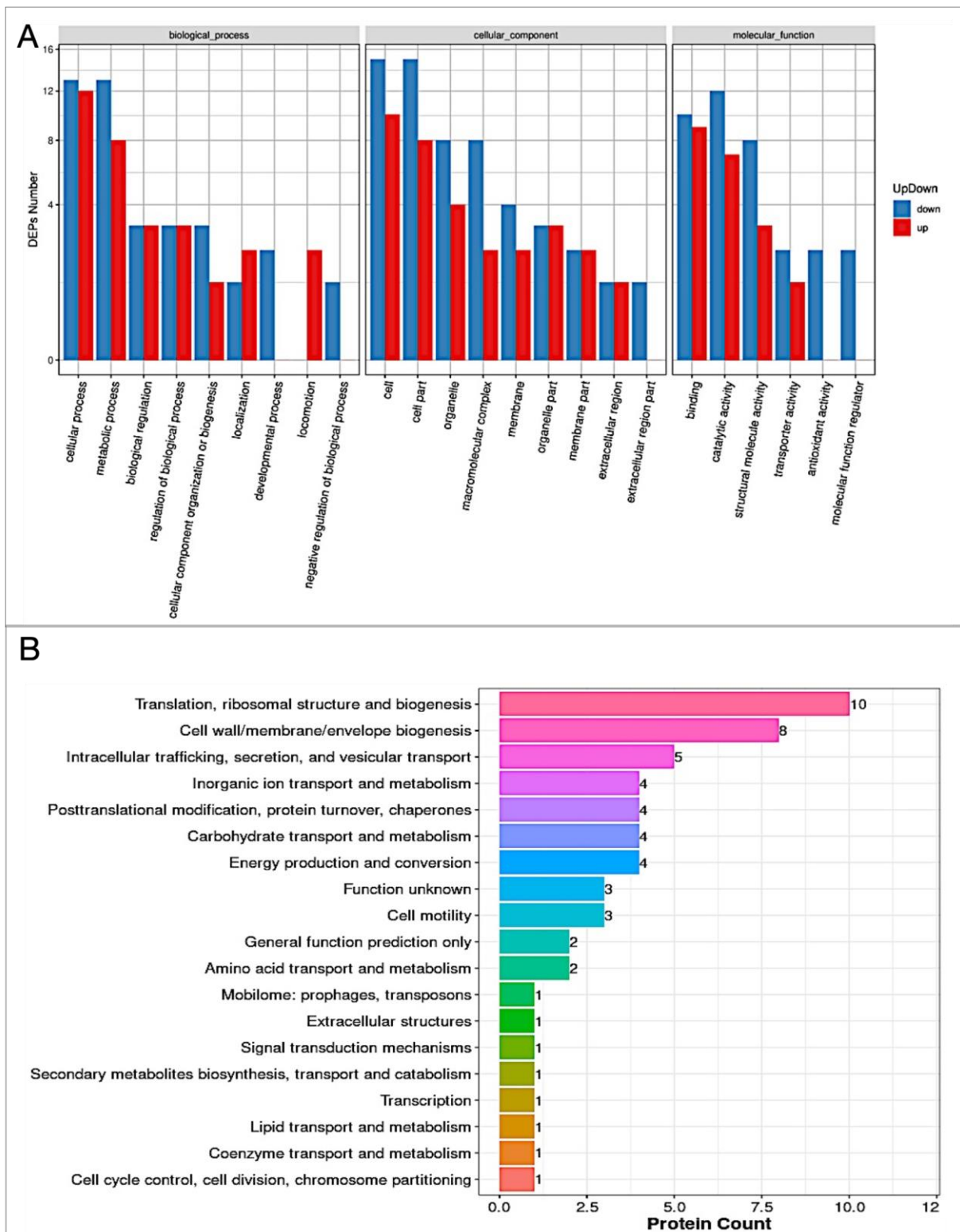


Figure 3.3: Differentially secreted protein (DSP) functions. **(A)** The graph shows gene ontology (GO) classes and annotation entries assigned to the upregulated (red) and downregulated (blue) proteins in the type 6 secretion system (T6SS) inactive secretome contrasted with the T6SS-active secretome. Each GO annotation entry is plotted against the number of associated DSPs. **(B)** The chart shows the number of

DSPs and the associated functional clusters of orthologous groups (COGs). The COG entries are plotted against the number of proteins associated with each entry.

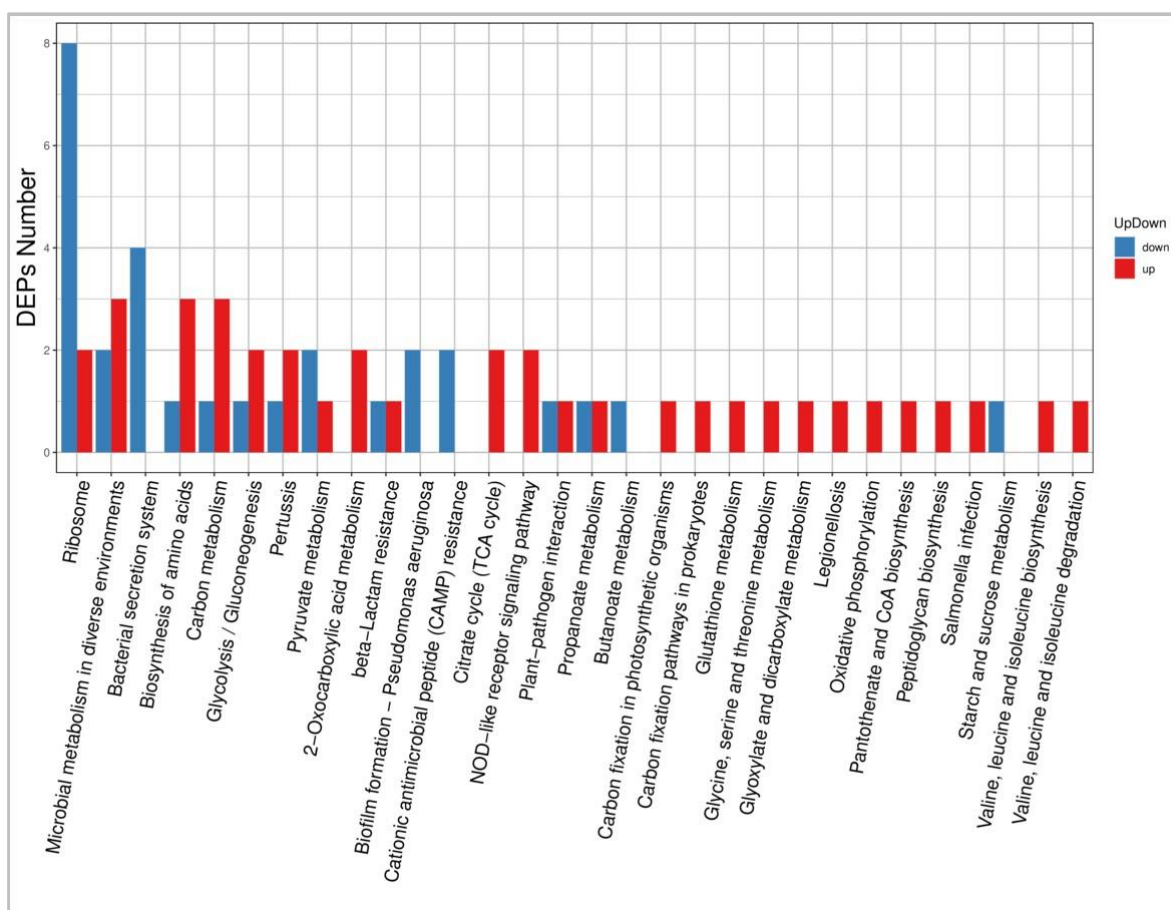


Figure 3.4: Differential protein pathway classification of type 6 secretion system (T6SS) inactive versus T6SS-active strain secreted proteins. The graph represents the pathway annotation entries against the number of differential proteins with up- or downregulation.

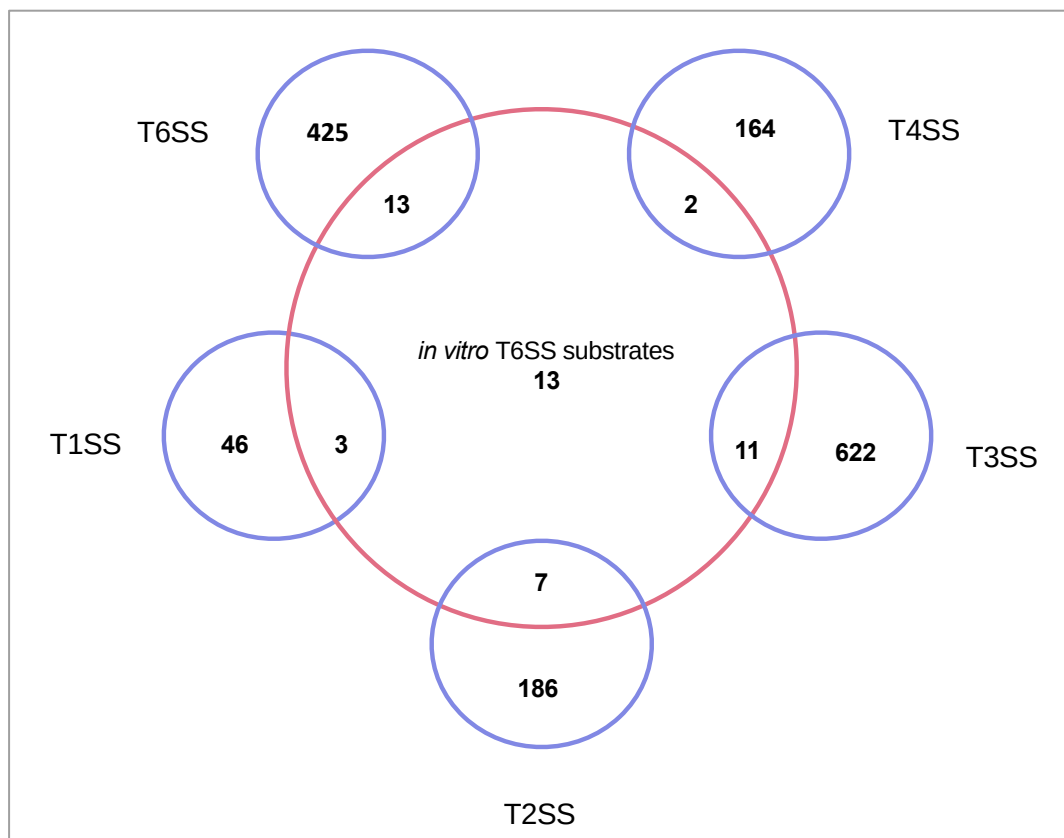


Figure 3.5: Potentially shared and exclusive *in silico* predicted secretion systems substrates and *in vitro* secreted type 6 secretion system (T6SS) substrates. The Venn diagram shows the number of the shared and primary substrates of secretion systems type 1 secretion system - type 4 secretion system (T1SS-T4SS) and T6SS predicted via BastionX, and the proteomics identified T6SS substrates *in vitro*. Thirty-five proteins are shown to be shared between the *in vitro* T6SS substrates and the substrates predicted for secretion systems T1SS-T4SS and T6SS.

Table 3.1. Predicted type 6 secreted substrates at >0.9 probability scores.

Protein name	Protein ID	T1	T2	T3	T4	T6	SS	SP	VF	CAZy
Avirulence protein L	tr A0A6I6X1F3 A0A6I6X1F3_9GAMM	0.342	0.91	0.095	0.172	0.965	T6SS	Y	N	N
Uncharacterised protein	tr A0A6I6X7F3 A0A6I6X7F3_9GAMM	0.489	0.867	0.196	0.408	0.983	T6SS	Y	N	N
TonB-dependent siderophore receptor	tr A0A6I6WU99 A0A6I6WU99_9GAMM	0.525	0.959	0.598	0.394	0.972	T6SS	Y	Y	N
Glycosyl hydrolase	tr A0A086ELY0 A0A086ELY0_9GAMM	0.335	0.781	0.508	0.389	0.962	T6SS	N	Y	Y
TonB-dependent siderophore receptor	tr A0A6I6WXM4 A0A6I6WXM4_9GAMM	0.648	0.961	0.666	0.339	0.962	T6SS	Y	Y	N
Maltoporin	tr A0A0M2F129 A0A0M2F129_9GAMM	0.725	0.948	0.63	0.409	0.957	T6SS	Y	Y	N
Pectate lyase	tr A0A0M2F0Z0 A0A0M2F0Z0_9GAMM	0.72	0.955	0.522	0.416	0.956	T6SS	Y	N	Y
Nitrate reductase (quinone)	tr A0A3S1AG98 A0A3S1AG98_9GAMM	0.16	0.831	0.161	0.207	0.954	T6SS	N	Y	N
Uncharacterised protein	tr A0A6I6XCF6 A0A6I6XCF6_9GAMM	0.438	0.57	0.581	0.489	0.953	T6SS	N	N	N
Family glycosylhydrolase	tr A0A6I6X3D0 A0A6I6X3D0_9GAMM	0.31	0.84	0.18	0.235	0.952	T6SS	Y	N	Y
YjbH domain containing protein	tr A0A6I6WZW3 A0A6I6WZW3_9GAMM	0.369	0.938	0.183	0.236	0.951	T6SS	Y	Y	N

Aldose 1-epimerase	tr A0A6I6WZW7 A0A6I6WZW7_9GAMM	0.194	0.873	0.667	0.354	0.948	T6SS	N	N	N
Pectin acetyl esterase	tr A0A6I6X513 A0A6I6X513_9GAMM	0.246	0.825	0.815	0.48	0.945	T6SS	Y	N	Y
Alpha-glucosidase	tr A0A6I6X094 A0A6I6X094_9GAMM	0.196	0.764	0.291	0.197	0.939	T6SS	N	N	Y
Type VI secretion stem protein VgrG tip	tr A0A6I6XDA3 A0A6I6XDA3_9GAMM	0.293	0.528	0.551	0.456	0.937	T6SS	N	Y	N
DUF3748 domain-containing protein	tr A0A6I6X3V3 A0A6I6X3V3_9GAMM	0.28	0.722	0.613	0.266	0.935	T6SS	Y	N	N
Porin	tr A0A0M2F7T5 A0A0M2F7T5_9GAMM	0.804	0.904	0.576	0.523	0.934	T6SS	Y	N	N
Alpha, alpha-phosphotrehalase	tr A0A6I6X0Y9 A0A6I6X0Y9_9GAMM	0.159	0.888	0.257	0.633	0.934	T6SS	Y	Y	Y
ShlB/FhaC/HecB family hemolysin secretion/activation protein	tr A0A0M2F1F4 A0A0M2F1F4_9GAMM	0.332	0.885	0.262	0.345	0.933	T6SS	Y	Y	N
Sulfatase-like hydrolase/transferase	tr A0A6I6X0S3 A0A6I6X0S3_9GAMM	0.152	0.794	0.124	0.453	0.932	T6SS	Y	Y	N
Sulfatase-like hydrolase/transferase	tr A0A6I6X124 A0A6I6X124_9GAMM	0.083	0.741	0.407	0.17	0.931	T6SS	Y	Y	N
Pectinesterase	tr A0A6I6X097 A0A6I6X097_9GAMM	0.331	0.919	0.302	0.215	0.931	T6SS	N	N	Y

DUF1565 domain-containing protein	tr A0A094QQ01 A0A094QQ01_9GAMM	0.884	0.916	0.6	0.185	0.93	T6SS	Y	N	Y
Type VI secretion system protein VgrG tip	tr A0A6I6X0F9 A0A6I6X0F9_9GAMM	0.217	0.347	0.229	0.321	0.925	T6SS	N	Y	N
Carbohydrate porin	tr A0A6I6XAQ4 A0A6I6XAQ4_9GAMM	0.513	0.92	0.496	0.476	0.925	T6SS	Y	N	N
Beta-galactosidase	tr A0A6I6XBR0 A0A6I6XBR0_9GAMM	0.125	0.754	0.228	0.114	0.924	T6SS	N	N	Y
Catalase	tr A0A6I6X3D4 A0A6I6X3D4_9GAMM	0.225	0.722	0.695	0.586	0.923	T6SS	Y	Y	N
5-deoxy-glucuronate isomerase	tr A0A6I6WWU9 A0A6I6WWU9_9GAMM	0.16	0.676	0.297	0.426	0.919	T6SS	N	N	N
Alpha, alpha-phosphotrehalase	tr A0A6I6XD12 A0A6I6XD12_9GAMM	0.109	0.717	0.188	0.258	0.918	T6SS	N	Y	Y
Peptide methionine sulfoxide reductase MsrA	tr A0A0M2EYW4 A0A0M2EYW4_9GAMM	0.166	0.444	0.349	0.408	0.916	T6SS	N	Y	N
Type VI secretion system tube protein Hcp	tr A0A6I6X5V0 A0A6I6X5V0_9GAMM	0.25	0.448	0.423	0.649	0.914	T6SS	N	Y	N
ShlB/FhaC/HecB family hemolysin secretion/activation protein	tr A0A6I6XD79 A0A6I6XD79_9GAMM	0.63	0.828	0.519	0.321	0.913	T6SS	Y	Y	N
DUF481 domain-containing protein	tr A0A6I6XBY8 A0A6I6XBY8_9GAMM	0.686	0.903	0.44	0.392	0.913	T6SS	Y	N	N

Oligopeptide ABC transporter substrate-binding protein OppA	tr A0A6I6XC98 A0A6I6XC98_9GAMM	0.2	0.886	0.293	0.625	0.911	T6SS	Y	N	N
Sulfatase-like hydrolase/transferase	tr A0A6I6WPM9 A0A6I6WPM9_9GAMM	0.21	0.762	0.197	0.304	0.91	T6SS	Y	Y	N
Oligogalacturonate lyase	tr A0A086F001 A0A086F001_9GAMM	0.353	0.57	0.272	0.428	0.906	T6SS	N	N	Y
Thioredoxin	tr A0A6I6X1P4 A0A6I6X1P4_9GAMM	0.282	0.838	0.161	0.203	0.905	T6SS	N	N	N
Family 43 glycosylhydrolase	tr A0A6I6X4L0 A0A6I6X4L0_9GAMM	0.164	0.743	0.422	0.492	0.901	T6SS	N	N	Y
Esterase family protein	tr A0A6I6X1J3 A0A6I6X1J3_9GAMM	0.214	0.758	0.234	0.379	0.901	T6SS	Y	Y	N
TonB-dependent receptor plug domain containing protein	tr A0A6I6X1C9 A0A6I6X1C9_9GAMM	0.872	0.992	0.752	0.756	0.964	T2SS	Y	Y	N
F5/8 type C domain-containing protein	tr A0A6I6X1Y3 A0A6I6X1Y3_9GAMM	0.835	0.991	0.779	0.744	0.909	T2SS	Y	N	N
Ferric-rhodotorulic acid/ferric-coproduct receptor FhuE	tr A0A6I6WNC8 A0A6I6WNC8_9GAMM	0.838	0.99	0.298	0.46	0.933	T2SS	Y	Y	N
TonB-dependent receptor	tr A0A6I6WNB3 A0A6I6WNB3_9GAMM	0.825	0.989	0.778	0.728	0.972	T2SS	Y	Y	N

TonB-dependent siderophore receptor	tr A0A6I6X1R8 A0A6I6X1R8_9GAMM	0.648	0.987	0.583	0.611	0.944	T2SS	Y	Y	N
Autotransporter domain-containing protein	tr A0A6I6XE67 A0A6I6XE67_9GAMM	0.814	0.985	0.647	0.573	0.913	T2SS	Y	Y	N
LPS-assembly protein LptD	tr A0A6I6X486 A0A6I6X486_9GAMM	0.527	0.985	0.419	0.377	0.96	T2SS	Y	N	N
PapC/FimD family outer membrane usher protein	tr A0A6I6WT06 A0A6I6WT06_9GAMM	0.571	0.985	0.453	0.44	0.95	T2SS	Y	Y	N
Endoglucanase	tr A0A6I6X0L8 A0A6I6X0L8_9GAMM	0.856	0.985	0.379	0.42	0.91	T2SS	Y	N	Y
Carbohydrate porin	tr A0A6I6WYS7 A0A6I6WYS7_9GAMM	0.713	0.979	0.303	0.334	0.955	T2SS	Y	N	N
Phage tail protein	tr A0A6I6X7Z6 A0A6I6X7Z6_9GAMM	0.779	0.976	0.544	0.418	0.948	T2SS	N	N	N
TonB-dependent siderophore receptor	tr A0A6I6X0P1 A0A6I6X0P1_9GAMM	0.511	0.975	0.26	0.365	0.943	T2SS	Y	Y	N
TonB-dependent siderophore receptor	tr A0A6I6WVT8 A0A6I6WVT8_9GAMM	0.51	0.974	0.516	0.381	0.905	T2SS	Y	Y	N
TonB-dependent receptor	tr A0A6I6X1J5 A0A6I6X1J5_9GAMM	0.695	0.973	0.755	0.569	0.958	T2SS	Y	Y	N
Vitamin B12 transporter BtuB	tr A0A6I6X4G9 A0A6I6X4G9_9GAMM	0.847	0.97	0.34	0.391	0.908	T2SS	Y	Y	N

TonB-dependent receptor	tr A0A6I6WT32 A0A6I6WT32_9GAMM	0.436	0.967	0.427	0.504	0.957	T2SS	Y	Y	N
TonB-dependent hemoglobin/transferrin/lactoferrin family receptor	tr A0A6I6WTR6 A0A6I6WTR6_9GAMM	0.654	0.965	0.641	0.322	0.945	T2SS	Y	Y	N
TonB-dependent siderophore receptor	tr A0A6I6WUQ6 A0A6I6WUQ6_9GAMM	0.699	0.964	0.81	0.664	0.911	T2SS	Y	Y	N
TonB-dependent hemoglobin/transferrin/lactoferrin family receptor	tr A0A6I6XDX8 A0A6I6XDX8_9GAMM	0.691	0.964	0.733	0.425	0.949	T2SS	Y	Y	N
TonB-dependent receptor	tr A0A6I6X1S0 A0A6I6X1S0_9GAMM	0.734	0.959	0.339	0.245	0.91	T2SS	Y	Y	N
Pectate lyase	tr A0A086ESF5 A0A086ESF5_9GAMM	0.731	0.958	0.613	0.505	0.936	T2SS	Y	N	Y
TonB-dependent receptor	tr A0A6I6X605 A0A6I6X605_9GAMM	0.87	0.957	0.681	0.698	0.921	T2SS	Y	Y	N
Pectate lyase	tr A0A6I6WY80 A0A6I6WY80_9GAMM	0.669	0.954	0.559	0.445	0.944	T2SS	Y	N	Y
Pectate lyase	tr A0A0M2F3M0 A0A0M2F3M0_9GAMM	0.765	0.949	0.526	0.377	0.917	T2SS	Y	N	Y
TonB-dependent receptor	tr A0A6I6WUC6 A0A6I6WUC6_9GAMM	0.687	0.944	0.6	0.429	0.924	T2SS	Y	Y	N

Pectinesterase	tr A0A0M2EZ13 A0A0M2EZ13_9GAMM	0.625	0.943	0.568	0.378	0.937	T2SS	Y	N	Y
Rhamnogalacturonan endolyase	tr A0A6I6XED8 A0A6I6XED8_9GAMM	0.398	0.943	0.166	0.224	0.936	T2SS	N	N	Y
TonB-dependent receptor	tr A0A6I6X7V5 A0A6I6X7V5_9GAMM	0.524	0.942	0.28	0.452	0.922	T2SS	Y	Y	N
TonB-dependent siderophore receptor	tr A0A6I6XBZ5 A0A6I6XBZ5_9GAMM	0.761	0.941	0.573	0.416	0.911	T2SS	Y	Y	N
Phospholipase A1	tr A0A6I6X3E8 A0A6I6X3E8_9GAMM	0.44	0.931	0.282	0.441	0.90	T2SS	Y	N	N
Arabinogalactan endo-beta-1,4-galactanase	tr A0A6I6X1L4 A0A6I6X1L4_9GAMM	0.627	0.923	0.246	0.27	0.901	T2SS	Y	N	Y

Key to Table:

Prediction Results based on Single Type Predictors

T1, Type 1 secretion system

T2, Type 2 secretion system

T3, Type 3 secretion system

T4, Type 4 secretion system

T6, Type 6 secretion system

Prediction Results based on Final Ensemble Model

SS, secretion system

T6SS, Type 6 secretion system

T2SS, Type 2 secretion system

SP, Signal Peptide

Y, Yes

N, No

VF, Virulence Factor

Y, Yes

N, No

CAZy, Carbohydrate-Active EnZyme

Y, Yes

N, No

Table 3.2. Type 6 secretion system active strain associated proteins identified by active and inactive strain secretome comparative proteomics.

Protein ID	T6SS-inactive strain			T6SS-active strain			Description	VFDB
Replicates	R1	R2	R3	R1	R2	R3		
tr A0A0M2F4K7 A0A0M2F4K7_9GAMM	0	0	0	2	4	3	SMI1/KNR4 family protein	N
tr A0A086E724 A0A086E724_9GAMM	0	0	0	1	0	3	Integration host factor subunit alpha	N
tr A0A0M2F514 A0A0M2F514_9GAMM	0	0	0	2	3	2	Flagellar L-ring protein	Y
tr A0A0M2F011 A0A0M2F011_9GAMM	0	0	0	0	1	2	2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase	N
tr A0A6I6X1N7 A0A6I6X1N7_9GAMM	0	0	0	0	1	2	Periplasmic serine endoprotease DegP-like	Y
tr A0A6I6XBK7 A0A6I6XBK7_9GAMM	0	0	0	13	10	5	HNH endonuclease	N
tr A0A0M2F5R1 A0A0M2F5R1_9GAMM	0	0	0	1	2	8	Hcp family type VI secretion system effector	Y
tr A0A6I6WLX1 A0A6I6WLX1_9GAMM	0	0	0	2	0	3	F5/8 type C domain-containing protein	N
tr A0A6I6WZR9 A0A6I6WZR9_9GAMM	0	0	0	4	2	3	YjbF family lipoprotein	N
tr A0A6I6X0F9 A0A6I6X0F9_9GAMM	0	0	0	7	5	7	Type VI secretion system tip protein VgrG	Y
tr A0A7V8Q6L5 A0A7V8Q6L5_9GAMM	0	0	0	3	3	4	Type VI secretion system tube protein Hcp	Y
tr A0A086EKP7 A0A086EKP7_9GAMM	0	0	0	1	0	2	50S ribosomal protein L19	N
tr A0A6I6X2I1 A0A6I6X2I1_9GAMM	0	0	0	4	2	2	Alpha/beta fold hydrolase	N
tr A0A086F0V0 A0A086F0V0_9GAMM	0	0	0	1	0	2	50S ribosomal protein L27	N
tr A0A0M2F1W7 A0A0M2F1W7_9GAMM	0	0	0	1	1	3	Two-component system QseEF-	N

							associated lipoprotein QseG	
tr A0A086EM34 A0A086EM34_9GAMM	0	1	0	18	18	14	Major exported protein	Y
tr A0A6I6X5V0 A0A6I6X5V0_9GAMM	0	0	0	3	3	3	Type VI secretion system tube protein Hcp	Y
tr A0A6I6X068 A0A6I6X068_9GAMM	0	0	0	3	2	2	Outer membrane channel protein TolC	Y
tr A0A6I6XDA3 A0A6I6XDA3_9GAMM	0	0	0	8	11	13	Type VI secretion system tip protein VgrG	Y

Key to Table:

Number of peptides identified in three different biological replicates of each strain (R1 to R3). 0, indicates that no peptides were detected.

VFDB, Homolog found in the virulence factor database

Y, Yes

N, No

Table 3.3. *In vitro* type 6 secretion system substrates and corresponding *in silico* predictions.

<i>In vitro</i> T6SS substrate ID	Protein name	Predicted secretion system	Probability score for predicted system	Probability score for T6SS secretion
tr A0A6I6X6T7 A0A6I6X6T7_9GAMM	Serralysin	T1SS	0.994	0.858
tr A0A6I6XD56 A0A6I6XD56_9GAMM	Flagellar protein FlhE	T2SS	0.523	0.336
tr A0A0M2F514 A0A0M2F514_9GAMM	Flagellar L-ring protein	T2SS	0.844	0.527
tr A0A6I6X068 A0A6I6X068_9GAMM	Outer membrane channel protein TolC	T3SS	0.858	0.554
tr A0A6I6X1N7 A0A6I6X1N7_9GAMM	Periplasmic serine endoprotease DegP-like	T3SS	0.547	0.189
tr A0A6I6WS59 A0A6I6WS59_9GAMM	TolC family protein	T3SS	0.863	0.355
tr A0A0M2F5R1 A0A0M2F5R1_9GAMM	Hcp family type VI secretion system effector	T6SS		0.887
tr A0A6I6X561 A0A6I6X561_9GAMM	Peptidyl-prolyl cis-trans isomerase	T6SS		0.575
tr A0A086EJ98 A0A086EJ98_9GAMM	Superoxide dismutase	T6SS		0.678
tr A0A6I6XDA3 A0A6I6XDA3_9GAMM	Type VI secretion system tip protein VgrG	T6SS		0.937
tr A0A086EM34 A0A086EM34_9GAMM	Major exported protein	T6SS		0.89
tr A0A6I6X2I1 A0A6I6X2I1_9GAMM	Alpha/beta fold hydrolase	T6SS		0.65
tr A0A6I6X0F9 A0A6I6X0F9_9GAMM	Type VI secretion system tip protein VgrG	T6SS		0.925
tr A0A086ET98 A0A086ET98_9GAMM	Hcp family type VI secretion system effector	T6SS		0.874
tr A0A6I6X5V0 A0A6I6X5V0_9GAMM	Type VI secretion system tube protein Hcp	T6SS		0.914

tr A0A6I6XBK7 A0A6I6XBK7_9GAMM	HNH endonuclease	T6SS		0.814
tr A0A6I6WZR4 A0A6I6WZR4_9GAMM	Alginate lyase	T6SS		0.87
tr A0A6I6X7E6 A0A6I6X7E6_9GAMM	Formate C-acetyltransferase	T6SS		0.584
tr A0A6I6WZU0 A0A6I6WZU0_9GAMM	Extracellular solute-binding protein	T6SS		0.74
tr A0A6I6X2J6 A0A6I6X2J6_9GAMM	Curli production assembly/transport component CsgG	T1SS	0.589	0.487
tr A0A086F0V0 A0A086F0V0_9GAMM	50S ribosomal protein L27	T1SS	0.512	0.499
tr A0A6I6XBV0 A0A6I6XBV0_9GAMM	Amidohydrolase family protein	T2SS	0.754	0.657
tr A0A0M2EZ13 A0A0M2EZ13_9GAMM	Pectinesterase	T2SS	0.943	0.937
tr A0A6I6WLX1 A0A6I6WLX1_9GAMM	F5/8 type C domain-containing protein	T2SS	0.989	0.883
tr A0A6I6X1C4 A0A6I6X1C4_9GAMM	Phospholipid-binding lipoprotein MlaA	T2SS	0.616	0.572
tr A0A6I6X0L8 A0A6I6X0L8_9GAMM	Endoglucanase	T2SS	0.985	0.91
tr A0A6I6XAY3 A0A6I6XAY3_9GAMM	Penicillin-binding protein activator LpoB	T3SS	0.631	0.435
tr A0A0M2EVG9 A0A0M2EVG9_9GAMM	50S ribosomal protein L29	T3SS	0.567	0.154
tr A0A0M2F1W7 A0A0M2F1W7_9GAMM	Two-component system QseEF-associated lipoprotein QseG	T3SS	0.951	0.734
tr A0A0M2F4K7 A0A0M2F4K7_9GAMM	Cell wall assembly protein	T3SS	0.708	0.516
tr A0A086EDQ7 A0A086EDQ7_9GAMM	50S ribosomal protein L18	T3SS	0.59	0.235

tr A0A6I6X1L8 A0A6I6X1L8_9GAMM	Penicillin-binding protein activator LpoA	T3SS	0.906	0.647
tr A0A0M2EW08 A0A0M2EW08_9GAMM	M48 family metalloprotease	T3SS	0.968	0.55
tr A0A086EFY4 A0A086EFY4_9GAMM	50S ribosomal protein L17	T3SS	0.558	0.128
tr A0A086E724 A0A086E724_9GAMM	Integration host factor subunit alpha	T4SS	0.578	0.171
tr A0A086EL99 A0A086EL99_9GAMM	DUF4810 domain-containing protein	T4SS	0.715	0.455

Supplementary Information

Table S3.1. Type 6 secretion system inactive strain associated proteins identified by active and inactive strain secretome comparative proteomics.

Protein ID	T6SS-inactive strain			T6SS-active strain			Description
Replicates	R1	R2	R3	R1	R2	R3	
tr A0A086E9V7 A0A086E9V7_9GAMM	4	4	2	0	0	0	Cold-shock protein
tr A0A7V8TL29 A0A7V8TL29_9GAMM	1	1	3	0	0	0	Glucose-6-phosphate isomerase
tr A0A7V8TME5 A0A7V8TME5_9GAMM	0	2	1	0	0	0	Purine-nucleoside phosphorylase
tr A0A433N8E0 A0A433N8E0_9GAMM	0	3	1	0	0	0	Glutamine synthetase
tr A0A0M2F4E6 A0A0M2F4E6_9GAMM	2	0	5	0	0	0	Fimbriae major subunit protein
tr A0A6I6X3Y3 A0A6I6X3Y3_9GAMM	1	0	7	0	0	0	Uncharacterised protein
tr A0A6I6WZ68 A0A6I6WZ68_9GAMM	5	5	4	0	0	0	Putative motility protein
tr A0A6I6WYH1 A0A6I6WYH1_9GAMM	2	2	1	0	0	0	Tail fiber assembly protein
tr A0A6I6X0F6 A0A6I6X0F6_9GAMM	0	1	4	0	0	0	Ail/Lom family outer membrane beta-barrel protein
tr A0A086E6K0 A0A086E6K0_9GAMM	4	3	1	0	0	0	Translation initiation factor IF-1
tr A0A6I6X3N2 A0A6I6X3N2_9GAMM	1	2	2	0	0	1	Uncharacterised protein
tr A0A6I6X9B7 A0A6I6X9B7_9GAMM	2	3	2	0	0	1	Uncharacterised protein

Key to Table:

Number of peptides identified in three different biological replicates of each strain (R1 to R3). 0, indicates that no peptides were detected.

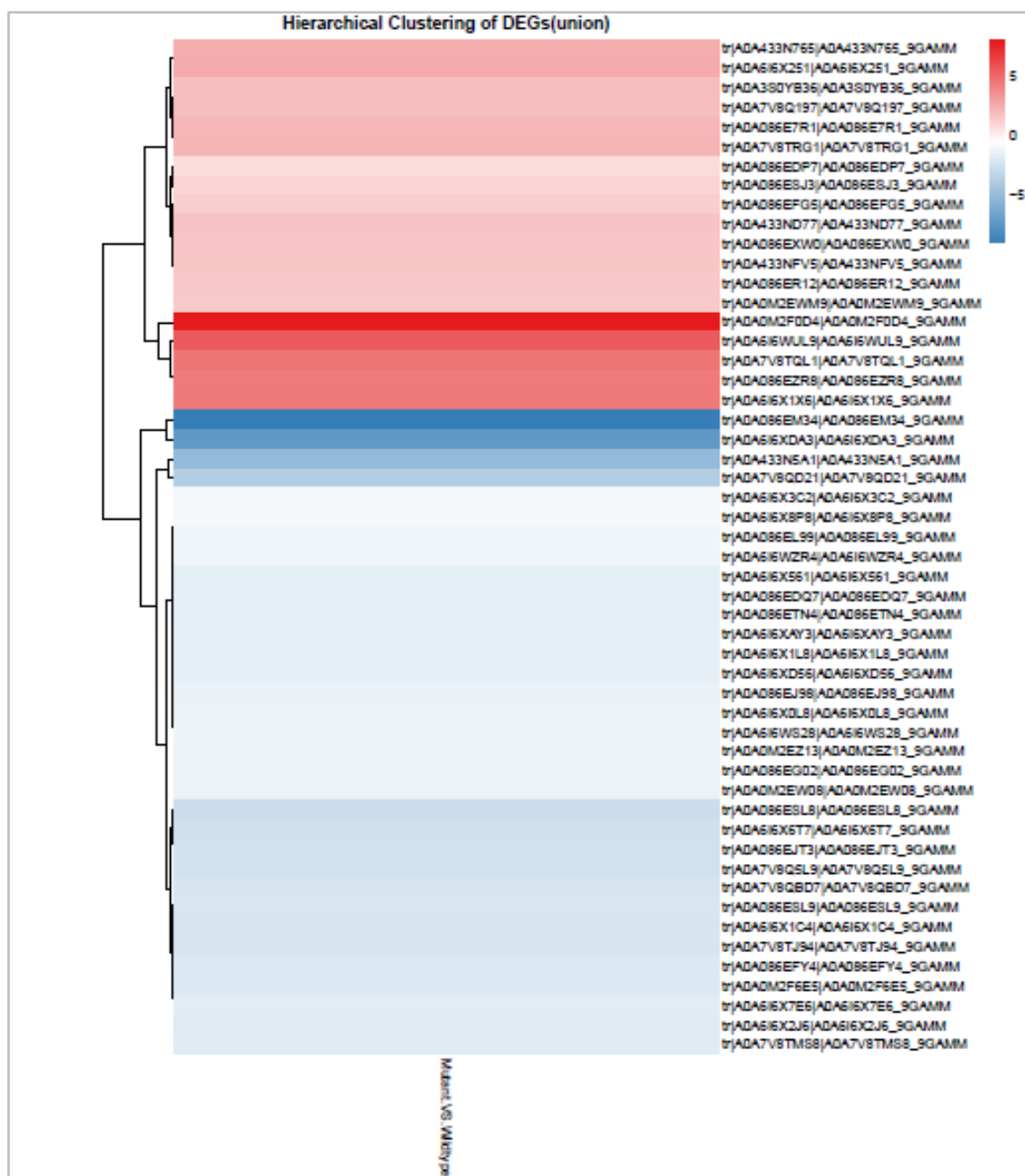


Figure S3.1: Differentially secreted proteins (DSPs) in the *Pectobacterium brasiliense* 1692 type 6 secretion system (T6SS) inactive strain secretome versus the T6SS-active secretome. The heatmap shows the 49 DSPs in the T6SS-inactive strain secretome in comparison to the T6SS-active strain secretome. Shades of blue show downregulated proteins and shades of red show upregulated proteins at a scale of -5 (blue) to 5 (red). Nineteen and 30 proteins were upregulated and downregulated respectively.

Table S3.2. Combined list of *in vitro* type 6 secretion system substrates identified by active and inactive strain secretome comparative proteomics.

Protein ID	Description	Type of substrate
tr A0A0M2F4K7 A0A0M2F4K7_9 GAMM	SMI1/KNR4 family protein	exclusive
tr A0A086E724 A0A086E724_9 GAMM	Integration host factor subunit alpha	exclusive
tr A0A0M2F514 A0A0M2F514_9 GAMM	Flagellar L-ring protein	exclusive
tr A0A0M2F011 A0A0M2F011_9 GAMM	2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase	exclusive
tr A0A6I6XBK7 A0A6I6XBK7_9G AMM	HNH endonuclease	exclusive
tr A0A0M2F5R1 A0A0M2F5R1_9 GAMM	Hcp family type VI secretion system effector	exclusive
tr A0A6I6WLX1 A0A6I6WLX1_9 GAMM	F5/8 type C domain-containing protein	exclusive
tr A0A6I6WZR9 A0A6I6WZR9_9 GAMM	YjbF family lipoprotein	exclusive
tr A0A6I6X0F9 A0A6I6X0F9_9G AMM	Type VI secretion system tip protein VgrG	exclusive
tr A0A7V8Q6L5 A0A7V8Q6L5_9 GAMM	Type VI secretion system tube protein Hcp	exclusive
tr A0A086EKP7 A0A086EKP7_9 GAMM	50S ribosomal protein L19	exclusive
tr A0A6I6X2I1 A0A6I6X2I1_9GA MM	Alpha/beta fold hydrolase	exclusive
tr A0A086F0V0 A0A086F0V0_9 GAMM	50S ribosomal protein L27	exclusive
tr A0A0M2F1W7 A0A0M2F1W7_9 GAMM	Two-component system QseEF-associated lipoprotein QseG	exclusive
tr A0A086EM34 A0A086EM34_9 GAMM	Major exported protein	exclusive
tr A0A6I6X5V0 A0A6I6X5V0_9G AMM	Type VI secretion system tube protein Hcp	exclusive
tr A0A6I6X068 A0A6I6X068_9G AMM	Outer membrane channel protein TolC	exclusive
tr A0A6I6XDA3 A0A6I6XDA3_9 GAMM	Type VI secretion system tip protein VgrG	exclusive
tr A0A6I6X1N7 A0A6I6X1N7_9G AMM	Periplasmic serine endoprotease DegP-like	exclusive
tr A0A6I6WS59 A0A6I6WS59_9 GAMM	TolC family protein	DSP
tr A0A6I6XBV0 A0A6I6XBV0_9G AMM	Amidohydrolase family protein	DSP
tr A0A0M2EVG9 A0A0M2EVG9_9 GAMM	50S ribosomal protein L29	DSP
tr A0A6I6X6T7 A0A6I6X6T7_9G AMM	Serralysin	DSP

tr A0A6I6XD56 A0A6I6XD56_9G AMM	Flagellar protein FlhE	DSP
tr A0A6I6X561 A0A6I6X561_9G AMM	Peptidyl-prolyl cis-trans isomerase	DSP
tr A0A086EJ98 A0A086EJ98_9G AMM	Superoxide dismutase	DSP
tr A0A6I6X2J6 A0A6I6X2J6_9G AMM	Curli production assembly/transport component CsgG	DSP
tr A0A0M2EZ13 A0A0M2EZ13_9 GAMM	Pectinesterase	DSP
tr A0A6I6X1C4 A0A6I6X1C4_9G AMM	Phospholipid-binding lipoprotein MlaA	DSP
tr A0A6I6X0L8 A0A6I6X0L8_9G AMM	Endoglucanase	DSP
tr A0A6I6XAY3 A0A6I6XAY3_9G AMM	Penicillin-binding protein activator LpoB	DSP
tr A0A086EDQ7 A0A086EDQ7_9 GAMM	50S ribosomal protein L18	DSP
tr A0A6I6X1L8 A0A6I6X1L8_9G AMM	Penicillin-binding protein activator LpoA	DSP
tr A0A0M2EW08 A0A0M2EW08_9 GAMM	M48 family metalloprotease	DSP
tr A0A086EFY4 A0A086EFY4_9 GAMM	50S ribosomal protein L17	DSP
tr A0A086EL99 A0A086EL99_9 GAMM	DUF4810 domain-containing protein	DSP
tr A0A6I6WZR4 A0A6I6WZR4_9 GAMM	Alginate lyase	DSP
tr A0A6I6X7E6 A0A6I6X7E6_9G AMM	Formate C-acetyltransferase	DSP
tr A0A6I6WZU0 A0A6I6WZU0_9 GAMM	Extracellular solute-binding protein	DSP
tr A0A086ESL8 A0A086ESL8_9 GAMM	50S ribosomal protein L4	DSP
tr A0A086ESN9 A0A086ESN9_9 GAMM	30S ribosomal protein S13	DSP
tr A0A6I6X3C2 A0A6I6X3C2_9G AMM	Superoxide dismutase	DSP
tr A0A0M2F6E5 A0A0M2F6E5_9 GAMM	Uncharacterised protein	DSP
tr A0A6I6WS28 A0A6I6WS28_9 GAMM	Uncharacterised protein	DSP
tr A0A086ETN4 A0A086ETN4_9 GAMM	50S ribosomal protein L28	DSP
tr A0A6I6X8P8 A0A6I6X8P8_9G AMM	Pyruvate kinase	DSP
tr A0A086EJT3 A0A086EJT3_9 GAMM	50S ribosomal protein L13	DSP
tr A0A086ESL9 A0A086ESL9_9 GAMM	50S ribosomal protein L23	DSP

tr A0A086ET98 A0A086ET98_9 GAMM	Hcp family type VI secretion system effector	DSP
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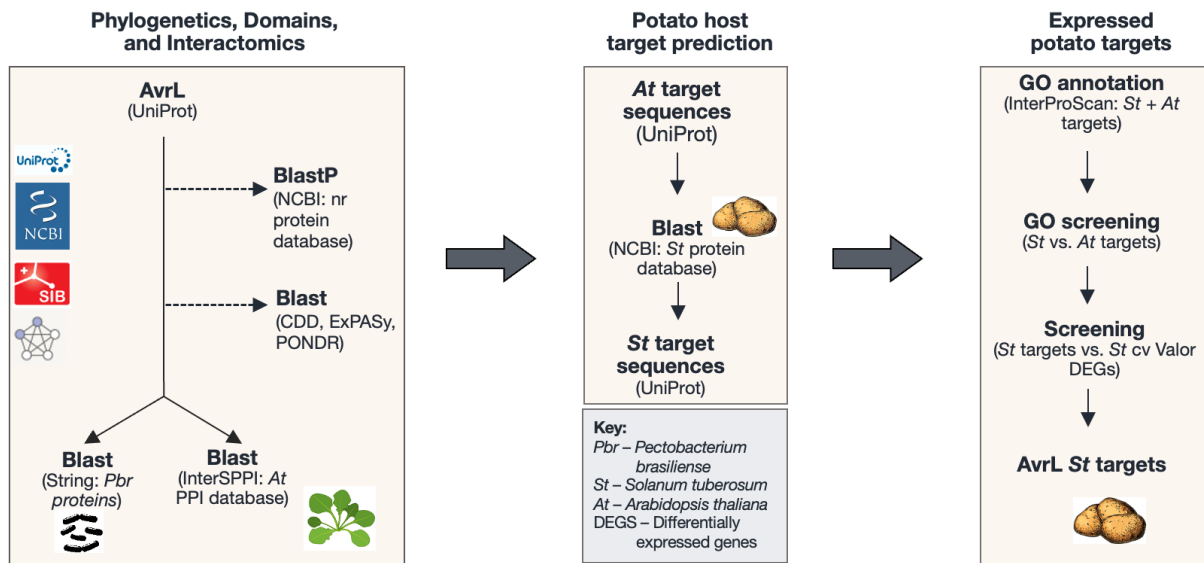
CHAPTER FOUR – *In silico* molecular interaction and characterisation of Avirulence protein L of *Pectobacterium brasiliense* 1692

Abstract

Avirulence-type effectors are typically secreted via the bacterial type 3 secretion system (T3SS). Based on the findings reported in Chapter Two of this thesis, Avirulence L (AvrL) forms part of the top ten most abundant cargo of *Pectobacterium brasiliense* 1692 (*Pbr*1692) outer membrane vesicles (OMVs) and might also be secreted via the type 6 secretion system (T6SS). The fact that this protein is potentially secreted via multiple channels suggests an important role in bacteria. Previous *Pbr*1692 RNA-seq data shows the upregulation of *avrL* during potato tuber infection, suggesting that AvrL might be involved in the virulence strategy of *Pbr*1692. The aim of this study was to provide more insights into the role of AvrL in the biology of *Pbr*1692 using computational analyses. Foremost, AvrL evolutionary relationships with its homologs show that it is highly conserved in 122 Soft Rot *Pectobacteriaceae* (SRP), with homologs having the conserved putative UDP-3-O-[3-hydroxymyristoyl] glucosamine N-acyltransferase domain belonging to the IpxD superfamily and a neutral metallopeptidase zinc binding region with the motif HExxH. Seven intrinsically disordered regions (IDRs) were also observed along the AvrL sequence. Using functional protein association network predictions, this study shows that ten *Pbr*1692 proteins, including plant cell wall degrading enzymes (PCWDEs), co-occur with AvrL. On the other hand, using an interspecies protein-protein interaction predictor, 150 *Solanum tuberosum* proteins were found to be potential AvrL targets, over 50% of which being transcription factors (TFs). Among these were TFs key in transcriptional reprogramming of plant gene expression in response to pathogen attack. Based on these findings, the study suggests that AvrL might be targeting some proteins involved in the defence responses of potato to *Pbr*1692 attack.

Keywords: Avirulence protein L, phylogenetics, functional domains, intrinsically disordered regions, host targets, transcriptional factors

Graphical abstract



Introduction

Secretion system studies are beset by an emerging yet complex expansion of repertoires of proteins systematically secreted into the extracellular environment or the target cells. Soft Rot *Pectobacteriaceae* (SRP) primarily employ the type 2 secretion system (T2SS) as the main virulence determinant, causing the soft rot phenotype observed on the hosts (Toth & Birch, 2005, Charkowski *et al.*, 2012). SRP harbor a large arsenal of plant cell wall degrading enzymes (PCWDEs), which are the typical T2SS substrates that break the cell wall (Chang *et al.*, 2014). For this reason, these bacteria are classified as necrotrophs. However, the necrotrophic nature of SRP is considered their dominant trait but not entirely exclusive of a biotrophic phase in their lifestyle. Thus, SRP can also exist as hemibiotrophs (Toth & Birch, 2005).

In addition to PCWDEs, the T2SS secretes other hydrolytic enzymes and molecules including lipases, proteases, necrosis-inducing protein (Nip), expansin YoaJ, putative virulence factor VirK, DUF 4879 family SvfA and SvfB, and Avirulence (Avr) proteins (Corbett *et al.*, 2005, Charkowski *et al.*, 2012, Condemine & Le Derout, 2022). Although the T2SS secretes some Avr proteins, Avr-type proteins in Gram-negative bacteria (GNB) are generally associated with the type 3 secretion system (T3SS), which secretes many effectors that facilitate host immune system subversion and manipulation (Lindeberg *et al.*, 2012, Peeters *et al.*, 2013, Piqué *et al.*, 2015, Roux *et al.*, 2015, Büttner, 2016).

The *avrL* of SRP is upregulated during *in planta* leaf and potato tuber infections in *Dickeya dadantii* 3937 and *Pbr1692*, respectively (Pédrón *et al.*, 2018, Bellieny-Rabelo *et al.*, 2019). Avirulence proteins associated with *Pectobacterium* spp. are AvrL, DspE/AvrE-family T3SS effector, and DspF/AvrF family protein (Holeva *et al.*, 2004, Corbett *et al.*, 2005, Kim *et al.*, 2011, Hogan *et al.*, 2013). DspF has been associated with the translocation and stability of DspE. Upregulation data for T3SS *dspF* and putative *dspE* was recorded for *D. dadantii* 3937 and *PccS1*, but not for *Pbr1692* (Pédrón *et al.*, 2018, Bellieny-Rabelo *et al.*, 2019, Fan *et al.*, 2020). *D. dadantii* grows as a bi-stable population where only a small fraction of the cells highly expresses some T3SS genes *in planta*, including *dspE* in the early stages (Cui *et al.*, 2018). A decrease in expression of *dspE* is observed after 12 hours post inoculation (hpi), showing lower levels at 24 and 48 hpi (Cui *et al.*, 2018). *avrL* is upregulated 24 hpi (Pédrón *et al.*,

2014, Bellieny-Rabelo *et al.*, 2019), meaning the protein AvrL might be one of the important effectors in the later stages of infection.

The T2SS non-pectinolytic AvrL was first identified as a homolog of *Xanthomonas campestris* AvrXca in *D. dadantii* 3937 (AvrL and AvrM) and in *P. atrosepticum* (Svx) (Parker *et al.*, 1993, Kazemi-Pour *et al.*, 2004, Corbett *et al.*, 2005). While *X. campestris* pv. *raphani* 1067 was avirulent to all *Arabidopsis thaliana* accessions infected, the transfer of the *avrXca* gene clone into the virulent strain *X. campestris* pv. *campestris* 8004 greatly reduced bacterial growth and symptom development in *A. thaliana* ecotype Columbia plants. In contrast, it caused virulence to ensue in *Brassica* plants. This shows that Avr effectors can be a liability detected by resistance proteins. However, their conservation is hinged on their benefits to the pathogen, wherein they evade the host immune recognition systems and promote virulence in compatible infections (successful infection leading to disease). In a characterisation study of Svx/AvrXca family virulence/avirulence protein, the protein was identified as a low abundance T2SS substrate involved in *in planta* virulence (Corbett *et al.*, 2005). The *svx* mutant strain displayed reduced virulence compared to the wild type (Corbett *et al.*, 2005). However, its full mechanism of action remains unknown. Protein database searches showed no functional information about Svx except that it had a zinc binding region signature (Corbett *et al.*, 2005). A study later showed that this M60-like zinc binding motif was mostly present in animal pathogen proteins, and only in *Pseudomonas syringae* proteins (Nakjang *et al.*, 2012). However, *P. syringae* proteins lacked extracellular features seen in other metallopeptidases in the same study (Nakjang *et al.*, 2012). Corbett and co-workers' (2005) hypothesis that Svx had extracellular enzymatic activity based on its possession of a signal peptide and the metallopeptidase zinc-binding motif (PF13402) has been validated recently (Tendiuk *et al.*, 2022).

Unlike most pathogens, support for the T3SS as one of the major virulence determinants of SRP is sparse (Kim *et al.*, 2009, Nykyri *et al.*, 2012, Davidson *et al.*, 2013). Perhaps even more puzzling is that some mutants and non-T3SS coding strains achieve the same effects as wild type strains (Charkowski *et al.*, 2012), suggesting that another system of secretion for these proteins is involved. Recently, our data implicated *Pbr1692*-derived outer membrane vesicles (OMVs) (Maphosa &

Moleleki, 2021), as we found AvrL to be abundant in these vesicles. Although OMV fusion to plant cells has not been widely shown, a few strategies have been proposed. For example, entry is suspected to take place when cells assume a relaxed state of the cell wall during cell growth, after recognition of OMV-associated microbe associated molecular patterns (MAMPs) (Bahar *et al.*, 2016). Alternatively, OMV lysis in the acidic apoplastic plant environment releases hydrolases that affect cell wall integrity (Solé *et al.*, 2015, Nascimento *et al.*, 2016). Thirdly, OMVs potentially enter the cell by fusion and internalisation via the clathrin dependent or independent mechanism (Rudnicka *et al.*, 2022). Although the fate of *X. campestris* pv. *campestris* derived OMV cargo is unknown, OMVs were observed to directly insert onto plant surfaces and interact with the plasma membrane (Tran *et al.*, 2022). With the above as background, we reasoned that AvrL could be transmitted from *Pbr1692* into host cells via OMVs, thereby assisting this pathogen in eliciting HR and/or evading host immunity via an undiscovered mechanism. Thus, OMVs appear to be an alternative strategy for secretions systems-dependent delivery of PCWDEs and virulence factors among other cargo (Bomberger *et al.*, 2009, Elhenawy *et al.*, 2014, Rompikuntal *et al.*, 2015, Solé *et al.*, 2015, Maphosa & Moleleki, 2021).

Avirulence L was also shown to be a putative T6SS substrate (Maphosa & Moleleki, 2021, thesis Chapters Two and Three). The T6SS is an injection system known for its primary role in competition (Cianfanelli *et al.*, 2016). The T6SS has other ecological roles including virulence and metal uptake (Chen *et al.*, 2016, Coulthurst, 2019, Chen *et al.*, 2020, Benavides *et al.*, 2021). Although the direct injection of T6SS effectors into plant cells has also not yet been demonstrated, the improved BastionX (<https://bastionhub.erc.monash.edu/> accessed on the 3rd of June 2022) predicts that the *Pbr1692* AvrL is a T2SS and T6SS substrate with a shared probability score of 1 (Wang *et al.*, 2021). Probability of secretion by T3SS was 0.575 which is above the ≥ 0.5 secretion cut off (Wang *et al.*, 2021). This implies that the AvrL could be a shared substrate with spatial roles including a potential role in host manipulation. Indeed, our previous transcriptomics data also indicated *avrL* among upregulated genes in *Pbr1692* 24 h and 72 h post infection of potato tubers, which signals there might be a role for this protein in pathogenesis (Bellieny-Rabelo *et al.*, 2019).

Avirulence-type effectors injected into host cells could benefit bacteria through hijacking of host defences. Bacterial effector host targets include defence related transcription factors (TFs) and the SKP1-CUL1-F-box protein (SCF) ubiquitin ligase complex. TFs are proteins that control diverse cell biological activities including plant development and defence (Li, 2015). In potato, four TF families, namely MYB, MYC2, AP2/ERF, and WRKY, were identified as part of the host response to *Pbr1692* infection (Kwenda *et al.*, 2016). Several studies indicate that Teosinte Branched1, Cycloidea, PCF1 (TCP) TFs play key roles in plant immunity and are effector targets (Mukhtar *et al.*, 2011, Weßling *et al.*, 2014, Yang *et al.*, 2017). Twenty-three TCPs are found in potato. Among these, *Solanum tuberosum* TCP23 was shown to be involved in the growth of potato and its defence regulation against bacterial pathogen *Streptomyces turgidiscabies* (Bao *et al.*, 2019). Like the MYC2 TFs, TCPs have a basic helix-loop-helix (bHLH) motif for DNA binding and protein-protein interactions (Heim *et al.*, 2003). Thus, effectors can bind TFs to inhibit transcriptional reprogramming and target them to the SCF complex for degradation. Bacteria such as *Agrobacterium tumefaciens*, *Ralstonia solanacearum*, and *Xanthomonas* spp. have been shown to encode SCF subunits known as F-box effectors that play an essential role in exploiting the host ubiquitin/proteasome system for pathogen benefit (Cunnac *et al.*, 2004, Tzfira *et al.*, 2004, Ji *et al.*, 2020).

Host cellular processes might also be hijacked using regions of protein backbones or intrinsically disordered regions (IDRs) that cannot assume a fixed tertiary structure (Uversky, 2013). IDRs are typically composed of evolutionarily conserved amino acids that define the diverse interactions with host proteins through eukaryotic linear motifs (ELMs), molecular recognition features (MoRFs), or short linear motifs (SLiMs) found within them (Mohan *et al.*, 2006, Diella *et al.*, 2008, Gould *et al.*, 2010). For example, SLiMs and post-translational modifications (PTMs) often influence protein-protein interactions and regulate protein function (Banfield, 2015, Popa *et al.*, 2016).

In this study, *Pbr1692* AvrL sequence analysis was used for phylogenetic assessment, which showed that AvrL is highly conserved in SRP. Using sequence analysis, AvrL functional domains were predicted in order to gain insight into their roles in *Pbr1692* biology and host-pathogen interaction. The computational analyses predicted that AvrL harbors two putative domains and IDRs. One domain was the previously

predicted zinc-binding region signature of neutral zinc metallopeptidases. The other was a putative domain which catalyses the third step of lipid A biosynthesis, hence is crucial for cell growth and survival. Next, *Pbr1692* proteins with functional associations with AvrL were predicted, with most of the functional partners identified to be T2SS substrates. This means that *Pbr1692* AvrL might participate in the degradation of the host cell wall minor extracellular protein component via the metallopeptidase domain. Lastly, an inter-species *A. thaliana* protein-protein interaction (PPI) network model was used as a template to predict AvrL interacting partners in potato host cells, confirming 150 proteins as potential AvrL targets. Ten differentially expressed TFs in response to infection by *Pbr1692* were identified among potential host targets in the susceptible *S. tuberosum* cv. Valor. These results suggest that AvrL interferes with host cellular biological processes including host defence response. Overall, the outputs of this study suggest that AvrL is a conserved multi-functional protein potentially involved in *Pbr1692* virulence.

Materials and methods

Sequence retrieval and phylogenetic analysis

The AvrL FASTA format protein sequence of *Pbr1692* (A0A6I6X1F3/WP_039319384.1) was retrieved from UniProt. The National Centre for Biotechnology Information (NCBI) (Madden, 2013) was used to perform a blast search against the NCBI nr database for AvrL homologs. The NCBI BlastP (protein-protein BLAST) algorithm was used to perform a query search against the NCBI nr database with $\geq 50\%$ sequence identity (e-value 0.01). A maximum likelihood phylogenetic tree was developed from AvrL and 393 AvrL homolog sequences aligned on MAFFT (Multiple Alignment using Fast Fourier Transform) version 7 (--auto) (Kato & Standley, 2013) and trimmed using trimAl on default parameters (Capella-Gutiérrez *et al.*, 2009) using IQTREE (Nguyen *et al.*, 2015). Ultrafast bootstrap and SH-aLRT values provided support for the tree branches and only values for the major branches were included. The tree annotation was performed using Figtree. Amino acid conservation of SRP AvrL proteins was evaluated using pairwise sequence similarity scores calculated by the Sequence Identities and Similarities (SIAS) server using the BLOSUM62 matrix (RechP, 2008). SRP protein sequences were also aligned and

visualised in a color-coded matrix display of the pair identity scores using the Sequence Demarcation Tool (SDT) (Muhire *et al.*, 2014).

AvrL physicochemical analysis

ProtParam tool in the ExPASy: SIB Swiss Institute of Bioinformatics resource was used to compute the physicochemical properties of AvrL (Gasteiger *et al.*, 2005).

AvrL conserved domains and intrinsically disordered regions prediction

Conserved domains were identified using the NCBI Conserved Domain Database (CDD) (Marchler-Bauer *et al.*, 2017) V3.19-58235 PSSMs database (e-value 0.01) and the ExPASy-PROSITE database (Sigrist *et al.*, 2012) with AvrL query sequence. The AvrL intrinsically disordered regions (IDRs) were identified using Predictor of Natural Disordered Regions (PONDR® VL-XT) (Romero *et al.*, 2004). VL-XT results are between 0 and 1, where 1 is the ideal prediction of disorder and 0 predicts order. A threshold value set to ≥ 0.5 strengthens disorder prediction.

AvrL function and functional partners prediction

The gene ontology (GO) term for AvrL was assigned using COFACTOR (Zhang *et al.*, 2017). STRING v11.5 (Szklarczyk *et al.*, 2019) was used to predict *Pbr1692* proteins that contribute to a shared function with AvrL.

AvrL model host plant targets prediction

Inter-Species Protein–Protein Interaction predictor (InterSPPI) version 3 AraPathogen tool was used to predict protein-protein interactions (PPIs) between the model host plant, *A. thaliana*, and AvrL. Acceptable interaction scores were above the prediction scores $>6.08\text{e-}06$ and specificity $\geq 97\%$ (Yang *et al.*, 2019). Descriptions of interacting proteins were retrieved from The *Arabidopsis* Information Resource (TAIR) tool (Lamesch *et al.*, 2012) using the gene model identifiers of the identified proteins. Functional annotation of the identified interacting proteins was performed using the Database for Annotation, Visualisation, and Integrated Discovery (DAVID) (Huang *et al.*, 2007). The DAVID output was summarised and visualised using the REVIGO webserver (Supek *et al.*, 2010) to show enriched proteins in the datasets as scatterplots of the gene ontologies. Duplicate or obsolete GO terms and terms with *p*-

values or False Discovery Rates [(FDRs) >0.50] were screened as non-significant entries and filtered out.

Identification of interactome homologs of *Arabidopsis* proteins in *Solanum*

The InterSPPI v3 Arapathogen webserver predicted interacting proteins of AvrL. FASTA format sequences were downloaded from UniProt and used in a NCBI BlastP search for homologous proteins in the *Pbr1692* host plant potato/*Solanum tuberosum* taxid: 4113 proteome (e-value 0.005, bit score ≥ 50). *S. tuberosum* reciprocal best hits (RBH) and the *A. thaliana* query interactomes were submitted to InterProScan for GO terms assignment (Jones *et al.*, 2014).

Screening of predicted *S. tuberosum* targets against transcriptome data profiles of *S. tuberosum* cultivars challenged with *Pbr1692*

Candidate potato targets were assigned potato genome sequencing consortium (PGSC) IDs and functionally enriched using g: Profiler online tool (Raudvere *et al.*, 2019). Transcriptome data profiles of *S. tuberosum* cv. BP1 and Valor generated by Kwenda, and coworkers (2016) were then used to screen InterSPPI v3 Arapathogen template predicted *S. tuberosum* targets to establish the potential interactors of AvrL expressed during infection by *Pbr1692*. The Plant Transcription Factor Database v5.0 (PlantTFDB) (Zhang *et al.*, 2011) was used to annotate transcription factor GO terms.

Results

AvrL homolog phylogeny

The *avrL* gene was reported to be upregulated *in planta* and involved in virulence, which means it might be an important protein in *Pectobacterium* spp. pathogenesis (Corbett *et al.*, 2005, Bellieny-Rabelo *et al.*, 2019). In this study, the aim was to establish the conservation status and function of AvrL in SRP. First, the maximum likelihood method was employed to perform evolutionary analysis and construct a phylogenetic tree of 393 homologs with $\geq 50\%$ sequence identity to AvrL (Figure 4.1). Branch lengths on the tree indicated the level of sequence divergence between the nodes or individual sequences (Figure 4.1). Phylogenetic reconstruction of the sequence evolutionary relationships revealed that AvrL homologs cluster into seven

distinct genera lineages (L1-L7). Among the lineages were SRP AvrL composed lineages L1, L3, and L4 (Figure 4.1). *Pbr1692* AvrL and all *Pectobacterium* spp. AvrL formed the L1 lineage. Other lineages (L2-L7) were of AvrL clusters of genera *Samsonia* (singleton), *Dickeya* (1;2), *Xanthomonas* (1;2), and *Zymobacter* (singleton) (Figure 4.1). *Pectobacterium* spp. AvrL were first identified as homologs of *Xanthomonas campestris* pv. *raphani* (Corbett *et al.*, 2005). *Pbr1692* AvrL is shown to share 57.99% sequence identity to the *Xca* virulent/avirulent protein. The results show genus specific clustering of AvrL homologs into seven phytopathogenic lineages that have diverged over time.

Further assessment of the SRP AvrL showed that the proteins shared $\geq 70\%$ sequence identity with *Pbr1692* AvrL. The SRP AvrL lineages showed that L1 further separates into six subgroups (A-F: Figure 4.2). The *Pbr1692* AvrL (WP_039319384.1) was identified in the largest L1 subgroup, A. The subgroup exhibited minimalistic variation among sequences based on branch lengths. Members of subgroup A included AvrL proteins from different species, such as *P. brasiliense*, *P. polaris*, *P. fontis*, *P. carotovorum* subsp. *carotovorum*, *P. carotovorum*, *P. parvum*, and *P. aquaticum*. Subgroup B members were identified as *P. parmentieri*, *P. wasabiae*, *P. betavascularum*, *P. zantedeschiae*, *P. punjabense*, and *P. atrosepticum*. Subgroup C was made up of *P. carotovorum* subsp. *carotovorum* and *Pectobacterium*. Subgroup D members were *P. carotovorum*, *P. versatile*, *P. parvum*, and *P. brasiliense*. Subgroups E and F were composed of *P. odoriferum* and *P. actinidae* respectively. *Dickeya* spp. clustered together to form L3 (Figure 4.2). L1 subgroups A-F showed diverse AvrL clustering with subgroup A and B forming the two major species diverse groups. *P. brasiliense* AvrL were all found in subgroup A, except for one in subgroup D. Overall, the results suggest that AvrL is highly conserved in SRP. In addition, the results imply that these proteins evolved from a common ancestor.

AvrL sequence conservation in SRP

Pairwise alignments were built to estimate the degree of conservation among SRP homologs at the amino acid residue level. Alignments of amino acid sequences of 30 proteins in the subgroup branches of the *Pectobacterium* lineage (L1) in SIAS server showed between ~88-99% exact residue matches with *Pbr1692* AvrL (data not shown). All against all pairwise alignments of all the 122 SRP AvrL protein sequences

including the *Samsonia* singleton AvrL were visualised in a SDT matrix heatmap. Figure 4.3 shows high sequence percentage identity between *Pectobacterium* spp. AvrL proteins (red) in all against all comparison of sequences from L1, L2, and L3. Red, yellow, and blue symbolise the highest, moderate, and lowest percentage identities between each pair of AvrL proteins, respectively. *Dickeya* against *Pectobacterium* sequence alignments showed the lowest (~70%) identity. Protein IDs and species details can be found in Figure S4.1. The results are in line with the tree stipulations (Figure 4.1 and 4.2) and imply that AvrL is highly conserved among SRP with sequence variations between the two SRP genera *Dickeya* and *Pectobacterium*. The conservation shown here further suggests that AvrL might be important for SRP in their ecological niches.

Pbr1692 AvrL physicochemical properties

The *Pbr1692* AvrL sequence was used to assess the protein's amino acid profile and other physical and chemical features. ExPASy-ProtParam was used to predict, in part, the biochemical function of AvrL and where it might be functionally stable inside the host cell subcellular compartments. It has been observed that the stable folded and functional states of proteins are often within pH values near their isoelectric points (pIs) that match specific subcellular localisations (Talley & Alexov, 2010). The predicted properties are shown in Table 4.2. In summary, the results show that AvrL is a stable protein of 621 aa with a molecular weight of 68.386 kDa. It has an almost equal number of negatively and positively charged residues and an alkaline pI of 7.66. While the apoplast or tuber pH where *Pbr1692* cells grow is acidic, in the initial stages of infection, pH levels increase to slightly basic (pH 8) by the late stage of infection (Grignon & Sentenac, 1991, Pérombelon, 2002, Kiszonas & Bamberg, 2010). Thus, these conditions appear adapted for AvrL extracellular activity. Moreover, pH in some host intracellular compartments such as the cytoplasm pH of ~7.5 also appears to support AvrL activity (Couldwell *et al.*, 2009).

SRP AvrL domains

Avirulence L's potential stability both outside and inside host cells was hinted at in the preceding section. In this perspective, sequence analysis was performed to predict that AvrL domains would be involved in the roles associated with *Pbr1692* proteins at

these sites. A CDD and PROSITE blast of *Pbr1692* AvrL was conducted to predict its conserved domains, families, and or patterns. A search for conserved domain footprints in AvrL using CDD showed that AvrL has a putative UDP-3-O-[3-hydroxymyristoyl] glucosamine N-acyltransferase domain (cl35126) that belongs to the lpxD superfamily between residues 485-567. These enzymes are used to synthesise lipid A, a crucial component of lipopolysaccharide in GNB. However, the *Pbr1692* AvrL lpxD domain was shown to have variations compared to the CDD lpxD domain hits. These variations were three >3 amino acid insertions, a single residue deletion, insertion, and substitution. While these were absent in the CDD profiles, they were conserved in all the SRP AvrL (Figure 4.4A). Notably, the insert DNARL is present in the conserved lpxD sequence. The conservation of the inserts and the repeat suggests a specific benefit to SRP. The function of the domain or benefit of the variations is not yet known.

All the *Pectobacterium* spp. AvrL (Figure 4.2) had the first consensus sequence, GTVR. However, *Dickeya* spp. had positively charged lysine (K) in place of uncharged threonine (T). The second sequence, DNARL, was also conserved in the *Pectobacterium* spp. AvrL. The *Dickeya* spp. AvrL exhibited polar uncharged glutamine (Q) instead of positively charged arginine (R) in the fourth position. The third sequence, PGAF, was conserved in both lineages with variations in a few *Pectobacterium* species, showing aromatic tyrosine (Y) instead of aromatic phenylalanine (F). Previously, the phylogenetic and residue similarity analysis showed that *Pectobacterium* and *Dickeya* spp. AvrL have approximately 70% sequence identity. The variations observed on the domain may have an effect on activity (Figure 4.4A).

ExPASy-PROSITE- identified *Pbr1692* AvrL as a zinc protease or neutral zinc metalloprotease with a zinc-binding region pattern at position 137-146. Amino acid sequence analysis of *Pectobacterium*, *Samsonia*, and *Dickeya* (L1-L3) AvrL showed that the zinc binding domain GLAHEFTHAL (Prosites PS00142) is strikingly conserved in all three lineages with only four proteins of *Pectobacterium* spp. having alanine (A) instead of threonine (T) (Figure 4.4B). Metalloproteases, especially those found in animal proteins, frequently contain the HEXxH zinc binding motif. The majority of these enzymes are important virulence factors that break down extracellular host

components during invasion, and are therefore, potential lucrative therapeutic targets (Duarte *et al.*, 2016, Hasan *et al.*, 2021). Metalloproteases and metallopeptidases belong to a zinc binding superfamily of metzincins based on sequence similarity. The monometallic HEXxH site was 100% conserved in the 121 SRP. The two domains predicted here suggest that the AvrL protein may play a role both in the producing parent bacteria and in the extracellular environment.

Pbr1692 AvrL intrinsically disordered regions

The metallopeptidase and lpxD superfamily domains are predicted to be present at two intervals along the AvrL protein sequence, according to the sequence analysis used in this study. These were not implicated in host immune system evasion or manipulation. Most structural and functional data for effectors often refers to folded domains of solved ordered proteins. In addition to completely folded regions of a protein, some effectors harbor IDRs that contain host protein binding motifs and microdomains whose structural 'flexibility' may influence effector translocation, evasion of the plant innate immune system, and host protein function mimicry (Marín *et al.*, 2013, Sámano-Sánchez & Gibson, 2020). To establish whether AvrL contains IDRs, this study analysed the AvrL sequence using PONDR VL-XT. AvrL was observed to harbor seven disordered regions in total along the 621 aa long protein sequence (Figure 4.5). The disorder was predicted to span the following residues 1-6, 121-127, 296-310, 400-404, 646-504, 570-577, and 599-611 where potential host-AvrL interaction could occur. The highest confidence PONDR prediction score of 0.8 was observed for the long, disordered region of 41 residues predicted between residues 464 and 504 (thick line, Figure 4.5).

Candidate *Pbr1692* AvrL functional partners and host target proteins

STRING was used to establish the molecular pathways and biochemical events involving *Pbr1692* AvrL. Our prior knowledge of AvrL in SRP suggested that it is a T2SS substrate (thesis Chapter Three, Kazemi-Pour *et al.*, 2004, Corbett *et al.*, 2005). Its functional partners in *Pbr1692* were determined to support its potential extracellular role in virulence. Based on co-occurrence or linkage across species genomes, PCWDEs including pectate lyases, rhamnogalacturonate lyase, and expansin YoaJ, two uncharacterised proteins, cytochrome C550, methyltransf-25 domain containing

protein, DltD, and sugar binding protein were predicted as partners of AvrL (Figure 4.6). The functional partners were predicted by co-occurrence at scores between 0.723 and 0.773. The network in Figure 4.6 shows that the group of 11 proteins are partially biologically connected. This prediction is in line with *P. atrosepticum* Svx secretion alongside some T2SS substrates (Corbett *et al.*, 2005). The results suggest the AvrL could have a shared function with T2SS substrates among other proteins. This has an implication for the conserved metallopeptidase domain in potential extracellular degradation of host cell protein components.

In Chapters Two and Three AvrL was found in *Pbr1692* OMVs and predicted to be a T6SS substrate, respectively. To determine the potential role of AvrL in plant cells, interactions with host intracellular proteins were predicted. Once inside the host cell, an effector protein can perform at least one of the following actions: inhibit PTI and/or ETI or induce HR. COFACTOR analysis in I-TASSER web server predicted that the function of AvrL is protein binding (GO:0005515). According to the description of the GO term, proteins annotated with this function interact selectively or non-covalently with other proteins and protein complexes, including non-protein molecules. The AvrL sequence was uploaded to the InterSPPIv3 online site in order to further investigate whether the AvrL protein interacts intracellularly with host proteins after delivery. The InterSPPI's AraPathogen predicted all potential protein-protein interactions (PPIs) between the model plant *A. thaliana* and phytopathogen *Pbr1692*, in this case via AvrL. To achieve this, the InterSPPI *A. thaliana* intra-species PPI network (AraPPI) containing 28110 PPIs of 7437 *A. thaliana* (*At*) proteins was screened against AvrL. Figure S4.2 shows a dense PPI network of the 152 proteins that were predicted to interact with AvrL. The predictions of the 152 proteins in order of highest possibility of interaction based on their probability prediction scores are shown in Table S4.1. A clearer visual on Cytoscape shows the number of interactions (protein hubs) of the top five most probable targets of AvrL in the host (Figure 4.7). The descriptions of the five and 20 other top predicted targets are shown in Table S4.2. In Figure 4.7, the interactions between the predicted targets imply that AvrL potentially interferes with cellular hubs involved in plant defence signaling.

Functional annotations of *Pbr1692* AvrL candidate interactors in *A. thaliana*

The Database for Annotation, Visualisation, and Integrated Discovery (DAVID) tool was used to describe the functions of the proteins that are potential targets of AvrL in model host plant *A. thaliana*. The annotations were made to predict the processes potentially under AvrL influence during host-pathogen interactions. A total of 94, 124, and 90 biological process, cellular component, and molecular function GO terms were assigned, respectively. GO terms regulation of defence response (GO:0031347), regulation of transcription, DNA templated (GO:0006355), transcription, NLS-bearing protein import into the nucleus (GO:0006607), and jasmonic acid-mediated signaling pathway (GO:0009867) were the most significantly enriched under biological process ontology. Out of 124 annotated proteins, cellular component GO terms nucleus (GO:0005634), and nuclear pore (GO:0005643) were the most significant in the ontology. In the molecular function ontology, the highly enriched and significant GO terms were protein binding (GO:0005515), transcription factor activity, sequence-specific DNA binding (GO:0003700), DNA binding (GO:0003677), nuclear localisation sequence binding (GO:0008139), and sequence-specific DNA binding (GO:0043565). The highly enriched GO terms were visualised using REVIGO (Figure S4.3). According to DAVID KEGG pathway annotations, 16 proteins were associated with the plant hormone signal transduction (8), ubiquitin-mediated proteolysis (4), and RNA transport (4) pathways.

Functional similarity between *A. thaliana* interactors and *S. tuberosum* proteins

Gene ontology term similarity was employed to screen predicted *A. thaliana* against *S. tuberosum* (potato) proteins. The screen was performed to provide provisional data for potato *Pbr1692* AvrL target screening using *S. tuberosum* cv. Valor and BP1 proteins. Sequences of the 152 proteins predicted in InterSPPI Arapathogen tool were retrieved from UniProt. Of these, 120 were manually reviewed (Swiss-Prot) (Mitchell *et al.*, 2019). NCBI BlastP was used to search for homologs of the predicted interactor *A. thaliana* proteins in *S. tuberosum* (St). Bit scores that were ≥ 50 were used to infer homology. Only two proteins had no significant hits. Among homologs, any *A. thaliana*-*S. tuberosum* homolog pair was identified as functionally similar if they shared ≥ 1 GO terms. All assigned homolog pairs shared at least one GO term, and this implies that the Arapathogen template is a good fit for interactome prediction of *Pbr1692* AvrL host

potato targets. Among the 150 potato candidates, 144 potatoes proteins were assigned potato IDs using the g:Convert (Gene ID conversion) platform in the g:Profiler tool. Functional enrichment analysis of the candidate potato host targets was also performed using g: Profiler web server via the g:GO St (Functional profiling) tool (Table S4.3). In total 1506, 471, and 375 proteins were assigned biological function, cellular component, and molecular functions respectively (Table S4.3).

Pbr1692 AvrL targets expressed during potato infection

It is essential to establish that the predicted target interactor proteins of a pathogen are expressed by the host during infection. Plant host expression in response to infection by *Pbr1692* between 0 and 72 h has been investigated in a tolerant *S. tuberosum* cv. BP1 and susceptible *S. tuberosum* cv. Valor (Kwenda *et al.*, 2016). AvrL likely interacts with proteins expressed during this period since it was shown to be upregulated in *Pbr*-potato *in planta* assays at 24 and 72 hpi (Bellieny-Rabelo *et al.*, 2019). An increase of DEGs between 24 and 72 h in both *S. tuberosum* cv. BP1 and *S. tuberosum* cv. Valor in comparison to controls was observed (Kwenda *et al.*, 2016). Thus, the availability of *Pbr1692* and potato transcriptome profiles provides a platform for comparison of strategies in infection and defence, respectively.

In this study, the potato cultivar profiles were employed to predict whether the candidate AvrL potato targets were expressed during *Pbr1692* infection. The AvrL targets were searched against the transcriptional profiles of the tolerant and susceptible potato cultivars to establish which of the putative 150 potato protein targets are expressed during infection. Comparison of the predicted candidate potato target set with expression profiles showed that 19 and 8 specific *S. tuberosum* cv. Valor and BP1 expressed genes match the AvrL predicted targets, respectively (Figure S4.4). In Valor, ten (53%) potential targets were transcriptional factors. One and seven out of the ten TFs were differentially expressed at 24 and 72 hpi, respectively (Kwenda *et al.*, 2016). The other potential targets were genes encoding two ubiquitin-protein ligases, kinesin light chain protein, three conserved proteins of unknown function, metal ion binding protein, exocyst complex component, and light-induced protein (Kwenda *et al.*, 2016). Thirteen of the 19 Valor genes were annotated 9 and 15 molecular function and biological ontology terms, respectively (Figure 4.8). None were assigned cellular component GO terms.

Among the eight BP1-specific genes were a transcription factor (TF), auxin-responsive protein, and ubiquitin carrier protein. Only two genes were assigned molecular functions. PGSC0003DMG400004012 was assigned nicotinate phosphoribosyltransferase activity (GO:0004516). PGSC0003DMG401000722 was assigned signaling receptor complex adaptor activity (GO:0030159) and signaling adaptor activity (GO:0035591). Fifteen proteins including a transcription factor, ubiquitin ligase, JA-induced WRKY protein, UP-9A, and several conserved genes of unknown function were identified targets in both BP1 and Valor cultivar profiles. However, none of the 15 could be assigned GO terms. Respectively, two and one conserved gene of unknown function were also identified among 3 and 2 intrinsic cultivar different genes of susceptible cv. Valor and BP1 (Kwenda *et al.*, 2016). These were shown to be present at time 0 hpi and important for plant growth and development (Kwenda *et al.*, 2016). Descriptions and corresponding target *S. tuberosum* PGSC IDs, *A. thaliana* (TAIR) IDs, and fold changes in gene expression are shown in Table S4.4. The results shown here imply that AvrL might target several potato proteins with biological and molecular functions in the host. The most represented targets are the TFs. Some plant TFs have been reported as major contributors to plant defence against pathogens, thus, are considered to be targets of effectors (Chacón-Cerdas *et al.*, 2020, Kwenda *et al.*, 2016). Table 4.3 shows the GO terms of the target *S. tuberosum* TFs according to the PlantTFDB. Cutin deficient 2, transcription factor 17, transcription factor (AtNAC domain-containing protein 89), and homeobox proteins have biological process GO terms regulation of defence response (GO:0031347), GO:0009827, response to salicylic acid (GO:0009751), response to jasmonic acid (GO:0009753), and plant-type hypersensitive response (HR) (GO:0009626) linked to defence response to pathogen attack.

Discussion

This study provides the first overview of the potential functional regions and functions of *Pbr1692* AvrL in the extracellular space and inside host cells. Extracellular proteins secreted via the T2SS such as AvrL are key in the pathogenesis and virulence strategies of SRP composed of *Pectobacterium* and *Dickeya* spp. (Charkowski *et al.*, 2012, Ma *et al.*, 2014). A recent study showed that, instead of plant cell death elicited by the T3SS, PCWDEs play a more significant role in *Pectobacterium* spp. induced

soft rot (Fan *et al.*, 2020). On the other hand, during host invasion by the pathogen, the plant harnesses its multilayered immune response to prevent or limit the harm caused by the secreted proteins (Nishad *et al.*, 2020, Nguyen *et al.*, 2021, Tanaka & Heil, 2021). Consequently, *Pectobacterium* spp. have evolved strategies and regulatory networks to persist in the toxic environment present in the host (Tanui *et al.*, 2017, Gorshkov *et al.*, 2018, Bellieny-Rabelo *et al.*, 2020, Fan *et al.*, 2020). Among these, we hypothesise that AvrL forms part of the virulence proteins delivered inside plant cells via OMVs or T6SS (Maphosa & Moleleki, 2021). In a recent study, *Pbr1692* AvrL and its homologs in other phytopathogens formed a clade distinctly separated from a clade of non-pathogenic bacteria (Tendiuk *et al.*, 2022). Thus, this work offers insights to the potential spatial molecular interactions of the AvrL protein with host proteins.

Important genes are generally conserved over evolutionary time scales. Therefore, our study set out to determine the functional significance of AvrL in SRP by confirming its conservation status. The evolutionary status of the AvrL protein suggested that the protein is conserved in *Pectobacteriaceae*. The *Pbr1692* AvrL was found in a *Pectobacterium* spp-specific lineage separate from other genera-specific lineages of *Dickeya*, *Xanthomonas*, *Samsonia* (singleton) and *Zymobacter* (singleton) (Figure 4.1). Within this lineage, the six subgroups showed minor sequence variations. The widespread of the AvrL protein was shown in 121 SRP encoding a *Pbr1692* AvrL protein homolog (Figure 4.2). In a previous study, all 84 studied *Pectobacterium* genomes were shown to possess the same AvrXca (svx) gene homologue (Li *et al.*, 2018).

The functional domain analyses of the AvrL protein suggested that the protein has putative UDP-3-O-[3-hydroxymyristoyl] glucosamine N-acyltransferase domain (Figure 4.4A). The lpxD enzyme is involved in the constitutive biosynthesis of lipid A, a component of lipopolysaccharides endotoxins required for the viability of almost all GNB. This role was first reported 29 years ago in *Escherichia coli* (Kelly *et al.*, 1993). Enzymes involved in lipid A biosynthesis have been considered attractive targets for developing new antibacterial agents (Raetz & Whitfield, 2002, Zhang *et al.*, 2013, Kroeck *et al.*, 2019). Considering that AvrL is targeted to the extracellular milieu and possibly host cells, it might exhibit acetyl-transferase activity inside host cells (Tasset

et al., 2010). However, it is not known if the variations observed in the SRP IpxD domain have functional implications. Notably, it was observed that part of the domain was within in the longest disordered region (Figure 4.5). IDRs are known to be involved in host system hijack by mimicry of host proteins. Thus, the activity of this domain is yet to be confirmed. The second domain was previously predicted by Corbett and coworkers in 2005. Similarly, this study identified *Pbr1692* AvrL as a metallopeptidase. The domain contains a zinc binding motif used by animal pathogens to degrade extracellular host components during invasion (Duarte *et al.*, 2016). It was predicted using ExPASy-PROSITE that AvrL possesses the same zinc-binding signature (Figure 4.4B). The *Pbr1692* AvrL homolog Svx's metallopeptidase domain targets α -glycosylated protein substrates of the plant cell wall (Tendiuk *et al.*, 2022). The assessment of the amino acid sequences of the AvrL homologs showed that the domain is conserved in SRP (Figure 4.2). This implies that the homologs have the same function.

Some proteins associate with other proteins or molecules towards a shared functional goal. In the extracellular environment, AvrL is predicted to associate/co-occur with several proteins including known T2SS-secreted pectinolytic proteins (Figure 4.6). It is also predicted to potentially interact with intracellular host proteins (Table S4.1). As a result, it could act as part of the brute force (aggressive) complex with exoenzymes that physically attack the host cell wall and induce damage associated molecular pattern (DAMP) triggered immunity. It can also be considered that the activity of PCWDE facilitates entry of co-occurring proteins such as AvrL. Alternatively, AvrL might act as a stealth factor (subtle), modifying host targets and manipulating plant immunity components to eventually intensify the aggression of necrotrophic *Pbr1692* (Mattinen *et al.*, 2004, Davidsson *et al.*, 2013, Hogan *et al.*, 2013). The AvrL's capacity to actively connect to its host targets without impairing its ability to associate with the pathogen protein network might be attributable to its mimicry of merely the host interaction modules (de Groot & Torrent Burgas, 2020). In addition to folds or domains, conserved residues in segments of intrinsically disordered polypeptides are known to be functional microdomains that can mediate functions comparable to structured domains (Gibson *et al.*, 2015). Through their disordered regions and motifs, proteins can interact and subdue the host via host protein mimicry and manipulation (Ronald & Joe, 2018, Lopez *et al.*, 2019, Sámano-Sánchez & Gibson, 2020). When under host

immune surveillance, mimicry of host proteins or slight changes in the effector eliminate the threat to cause effector triggered immunity (ETI). AvrL contains seven IDRs. The percentage disorder was recorded as moderately disordered at 15.30% (Ward *et al.*, 2004). This implies the presence of both ordered (structured/folded) and unstructured regions on AvrL suggesting it is a 'hybrid' protein (Dunker *et al.*, 2013). Thus, 15.30% of AvrL might mimic eukaryotic host activities, potentially tipping the scales of the evolutionary arms race between pathogen and host in favor of the pathogen. Henceforth, it was anticipated that the metallopeptidase domain, the IpxD superfamily domain, and/or the IDRs might be responsible for interaction with host proteins yet to be mined.

In Chapter Three it was predicted that the AvrL is a shared substrate of the T2SS and T6SS at probabilities 0.910 and 0.96, respectively. Of note, more recent modifications in the tool allow for different usage and mode options. The AvrL is currently predicted to be a shared T2SS and T6SS substrate (probability score = 1) (Wang *et al.*, 2021). Furthermore, it was previously discovered that *avrL*, alongside the T2SS and T6SS, was upregulated *in planta* (Bellieny-Rabelo *et al.*, 2019). The *Pbr1692 in planta* expression profile suggests that AvrL is important in the virulence of *Pbr1692* 24-72 hpi (Bellieny-Rabelo *et al.*, 2020). If by some unknown mechanism OMVs or T6SS deliver AvrL into the host it could potentially alter protein networks inside host cells. Also, a previous study of *P. atrosepticum* Svx a homolog of the AvrL protein, established that the mutant Svx strain exhibited reduced virulence in potato plant assays (Corbett *et al.*, 2005). The loss of virulence when the homolog of AvrL was mutated implies that the AvrL is important for *Pectobacterium* spp. virulence.

It was determined which of the host proteins of a model plant *A. thaliana* the AvrL protein likely interacts with should the AvrL and the host protein co-localise during infection. A total of 152 *A. thaliana* proteins that might interact with AvrL were identified. One hundred and fifty homologs with similar functions to the *A. thaliana* predicted candidates were identified in potato. It was seen that the functions associated with these proteins involve various host physiological processes. The expression profile of potato cultivars *S. tuberosum* cv. Valor and BP1 infected with *Pbr1692* served to screen the 150 putative targets (Kwenda *et al.*, 2016). Nineteen proteins were identified as targets in the susceptible *S. tuberosum* cv. Valor specific

DEGs. Most of these were TFs. Proteins containing IDRs in yeast *Saccharomyces cerevisiae* proteomes were shown to have the tendency to be localised the cell nucleus where they regulate transcription and cell signaling (Ward *et al.*, 2004). The study deduced that kinase activity and nucleic acid binding molecular activities relied directly on plasticity of the protein structure (Ward *et al.*, 2004). Bacterial effectors known as nucleomodulins likewise target the nucleus and hijack defence gene expression (Bierne & Cossart, 2012). *R. solanacearum* nucleomodulin Avr PopP2 has been shown to possess acetyl-transferase activity which has been proposed to offset host defence gene expression (Tasset *et al.*, 2010). It is hypothesised the *Pbr1692* AvrL UDP-3-O-[3-hydroxymyristoyl] glucosamine N-acyltransferase domain transferase activity could be useful in such host-pathogen interactions if active.

Effectors have also evolved to adapt to specific cellular environments to perform their functions. In *P. atrosepticum*, two forms of Svx were secreted. It was suspected that one form was a result of post translational modification (Corbett *et al.*, 2005). pIs of proteins correlate with their subcellular localisation (Talley & Alexov, 2010). It is plausible that the unmodified forms of the protein might be modified in the host which may alter its pI. Host-mediated post-translational effector modification may also facilitate the subcellular location and function of effectors (Popa *et al.*, 2016). Cytosolic proteins display basic pI (>7) and include TFs, 26S proteasome degradation proteins, auxin induced proteins, and extensins (Mohanta *et al.*, 2019). This implies that AvrL with a pI of 7.66 is stable in the plant cytosol. It is possible the AvrL targets and inhibits TFs in the cytoplasm preventing their activation and translocation for binding to DNA in the host cell nucleus. In the absence of active plant specific TCPs regulation of processes such as ETI defence responses by activating the biosynthetic pathways of bioactive metabolites, such as terpenoids, phytohormones, phenolic compounds, and pathogenesis related proteins are also inactivated.

In this study, several TFs were identified as targets of AvrL in *S. tuberosum* cv. Valor and only two in BP1 (Table S4.4). Avr-type effectors have been shown to manipulate host defences by binding TFs (Mukhtar *et al.*, 2011). Predicted *Pbr1692* AvrL target Lanceolate is a TCP3 ortholog that promotes flavonoid synthesis (Li & Zachgo, 2013). TCP14, a transcription regulator that contributes to ETI against biotrophs and hemibiotrophs by repressing some JA response genes is another effector target (Yang

et al., 2017). To divert attention from themselves, the biotrophic and hemibiotrophic pathogens bind TCP14 and target it to the SCF complex for degradation (Yang *et al.*, 2017). The repressed JA response genes are activated in its absence, and SA is repressed (Yang *et al.*, 2017). Toth and Birch (2005), several years ago, reviewed studies on *Pectobacterium* spp. and agreed that 'stealth force' (less aggressive) and 'brute force' qualities should be regarded as true non-mutually exclusive modes of pathogenesis when it comes to SRP and that the SRP are not as worlds apart from phytopathogens such as *P. syringae* (case in point defined as biotrophic, or hemibiotrophic, and occasionally necrotrophic or partially necrotrophic) in terms of mechanism of infection (Toth & Birch, 2005, Kraepiel & Barny, 2016). This suggests that AvrL could form part of the QS-regulated plant response suppressors of *Pectobacterium* spp. Therefore, there is a good reason to support this deduction based on the potential interaction of AvrL with another pathogen-induced transcriptional factor JA-induced WRKY protein in both *S. tuberosum* cv. Valor and BP1 and StTCP17 (*At*TCP13 homolog) in cv Valor. TF WRKY60 was shown to lead to *A. thaliana* disease susceptibility to both *P. syringae* and *Botrytis cinerea* (Xu *et al.*, 2006). *At*TCP13 was shown to be one of the direct targets of pathogen effectors (Mukhtar *et al.*, 2011). To note, StTCP17, in addition to being identified as a target in susceptible *S. tuberosum* cv. Valor was the second most probable target of AvrL among the predicted 152 *A. thaliana* proteins (Table S4.1, Table S4.2, Table S4.4). Figure 4.7 shows that *At*TCP13 has a network consisting of 419 other proteins. This suggests that targeting StTCP17 affects a large hub of proteins.

During host-pathogen interaction, bacteria's mechanisms of host targeting also include manipulation of the ubiquitin proteasome system (UPS). The UPS post-translationally modifies proteins by isopeptide bond formation to ubiquitin through a lysine side chain of the target to degrade, re-localise, alter its activity, or manipulate protein-protein interactions. Two ubiquitin-protein ligases were DEGs in *S. tuberosum* cv. Valor (Table S4.4). AvrL potential targets and interactions were predicted to influence activities that include protein ubiquitination and ubiquitin protein transferase activity (Figure S4.3). This suggests that the AvrL might be hijacking the UPS by mimicking the host SCF-type E3 ubiquitin ligase complex F box protein thus binding TFs or other targets and interacting with E3 ligases to mark the proteins for degradation. Effectors either bind to or act like E3 ligase enzymes to promote or hinder protein degradation (Büttner,

2016). E3 ubiquitin ligases have also been implicated in the modulation of plant immunity (Zhou & Zeng, 2017). Previously, an oomycete RxLR, Avr3a, and the *X. oryzae* pv. *oryzae* XopP were reported to interact with host ubiquitin E3 ligases to interfere with cell death (Bos *et al.*, 2010, Ishikawa *et al.*, 2014). In another example, *P. syringae* AvrPtoB was shown to suppress cell death and interact with tomato ubiquitin, resulting in its ubiquitination *in vitro* and show of E3 Ub ligase activity through which it targeted a pattern recognition receptor (PRR) for degradation (Abramovitch *et al.*, 2006, Göhre *et al.*, 2008). Similarly, *P. syringae* HopM1 targets an immunity-associated protein, AtMIN7 and facilitates its destruction via the host UPS (Nomura *et al.*, 2006). *X. campestris* pv. *vesicatoria* XopL has E3 ligase activity and induces plant cell death and subverts plant immunity (Singer *et al.*, 2013). F-box protein-like proteins also identified in *R. solanacearum*, *X. campestris*, and *A. tumefaciens* manipulate the host UPS (Remigi *et al.*, 2011, Üstün & Börnke, 2014, Lacroix & Citovsky, 2015). Hijacking the SCF-ubiquitin ligase system would be a significant strategy by *Pbr1692* to manipulate host defence and other cellular processes. The above suggests that AvrL may be important for bacterial suppression of host defences and induction of cell death. Both proteasome and autophagy pathways have been shown in several research as focal points of pathogen effectors where broader impacts on the host were observed. (Üstün & Börnke, 2014, Weßling *et al.*, 2014, González-Fuente *et al.*, 2020). As control centers of multiple facets of plant immunity that include receptor accumulation, pathogen recognition, and downstream defence signaling, it would be interesting if the AvrL targets them.

Altogether, 19 and 8 candidates for interaction with AvrL were identified in susceptible and tolerant potato cultivars, *S. tuberosum* cv. Valor and BP1, respectively. The next step of this study requires experimental validation of the interaction between AvrL and the putative interacting partners. For example, future assays will include Yeast 2 Hybrid (Y2H) assays as in the studies on plant interactors of effector proteins from *X. oryzae* pv. *oryzae*, *P. syringae*, and *Hyaloperonospora arabidopsidis* to ascertain interaction (Abramovitch *et al.*, 2006, Ishikawa *et al.*, 2014, González-Fuente *et al.*, 2020).

Conclusions

This study lays the groundwork for further exploration of the characteristics and likely roles of the AvrL virulence factor in *Pbr1692*'s interactions with its host plants. The study postulates that AvrL participates in lipid A biosynthesis in *Pbr1692* cells, is secreted via the T2SS to cause host cells to necrotise through the activity of the metallopeptidase domain and is also transported into host cells via OMVs and the T6SS, where it interacts with numerous host proteins like TFs. More reliable experimental research is anticipated to supplement the current computational analysis and screen for significant interactions to advance our understanding of the AvrL protein, whose role in SRP infection is still a mystery. There is still much to learn because, in general, it is difficult to comprehend pathogenic mechanisms for plant disease prevention and management.

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Figures and Tables

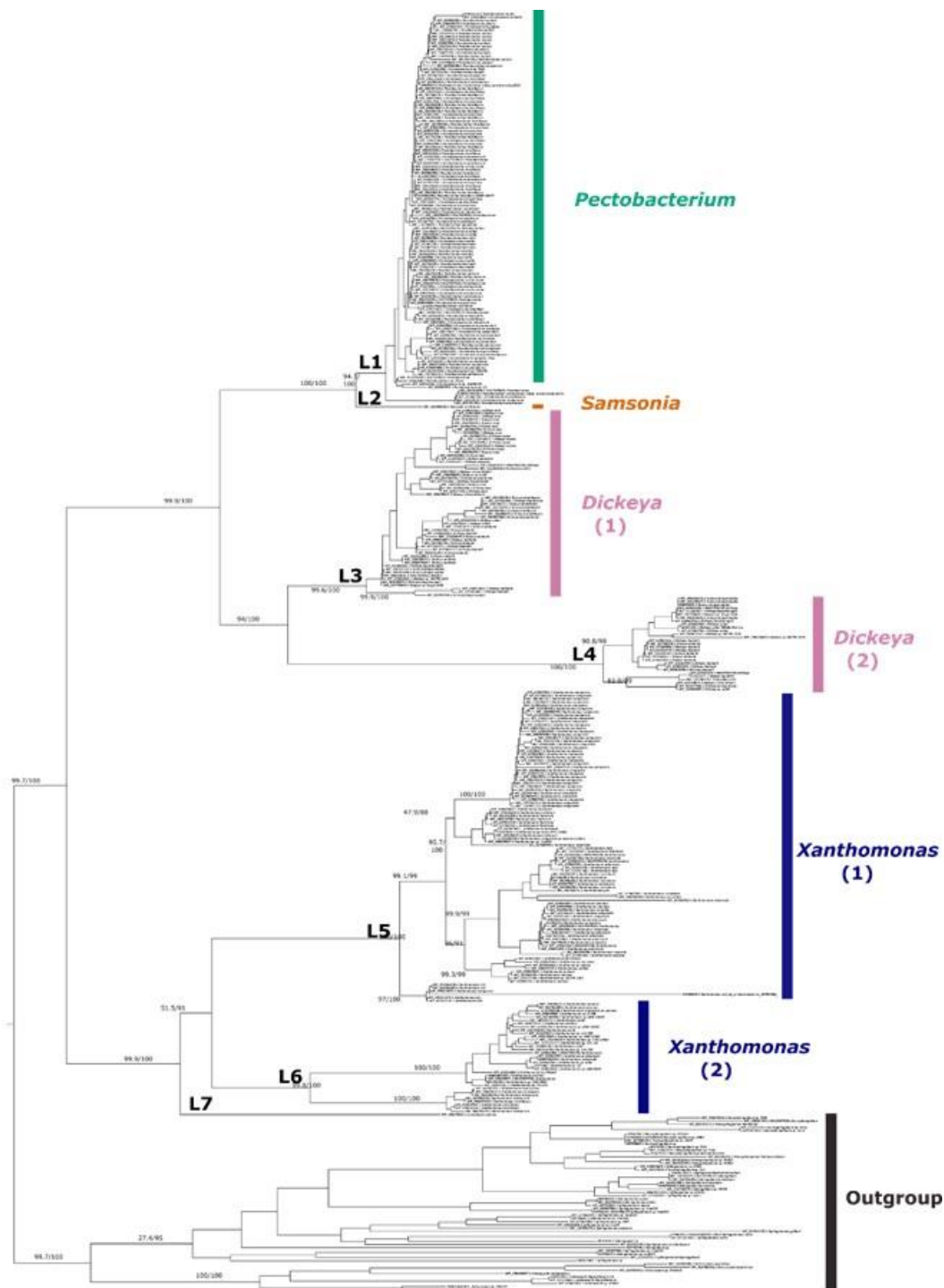


Figure 4.1: Maximum likelihood phylogenetic tree based on the amino acid sequences of 394 phytopathogenic bacteria AvrL proteins. The phylogenetic tree shows seven closely related but distinct clusters of AvrL proteins indicated as lineages (L1-L7) and their corresponding genera, namely, *Pectobacterium* (L1), *Samsonia* (L2), *Dickeya* (L3; L4), *Xanthomonas* (L5; L6), *Zymobacter* (L7), and an outgroup composed

of *Novosphingobium*, *Sphingomonas*, *Sphingobium*, and *Asticcacaulis* spp. Support values (Ultrafast bootstrap and SH-aLRT) calculated in IQTree are indicated at the nodes. The scale bar represents the number of amino acid changes per site.

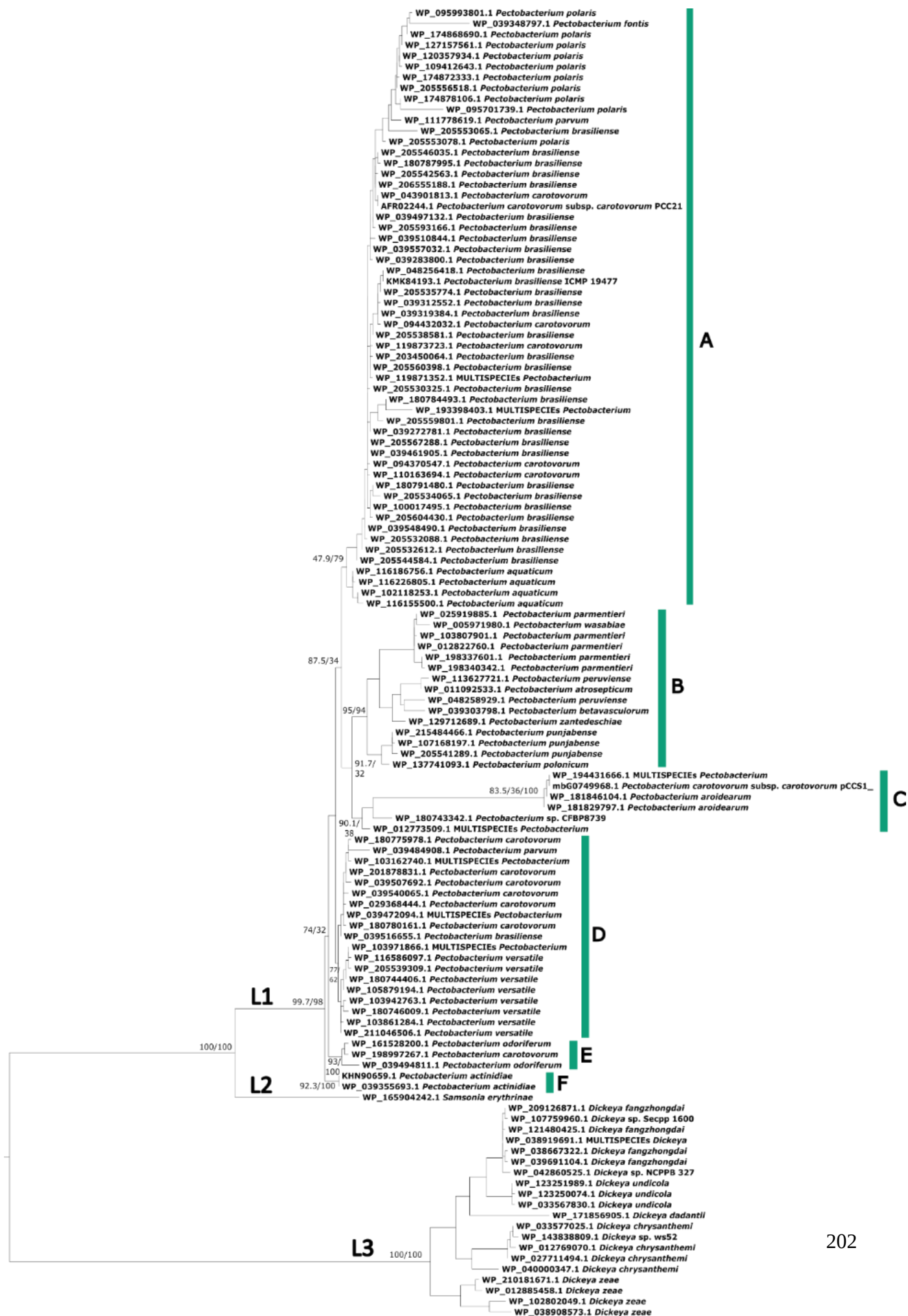


Figure 4.2: Maximum likelihood phylogenetic tree of 121 Avirulence L homologs with $\geq 70\%$ sequence identity to the *Pectobacterium brasiliense* 1692 Avirulence L protein. The phylogeny shows that the *Pbr*1692 AvrL homologs evolved from a common ancestor and belong to genera *Pectobacterium* (L1), one *Samsonia* (L2), and *Dickeya* (L3). *Pectobacterium* spp. (L1) have six subdivisions forming distinct groups (subgroups A-F) highlighted in green. Support values (Ultrafast bootstrap and SH-aLRT) calculated in IQTree are indicated at the nodes, and the scale bar represents the number of amino acid changes per site.

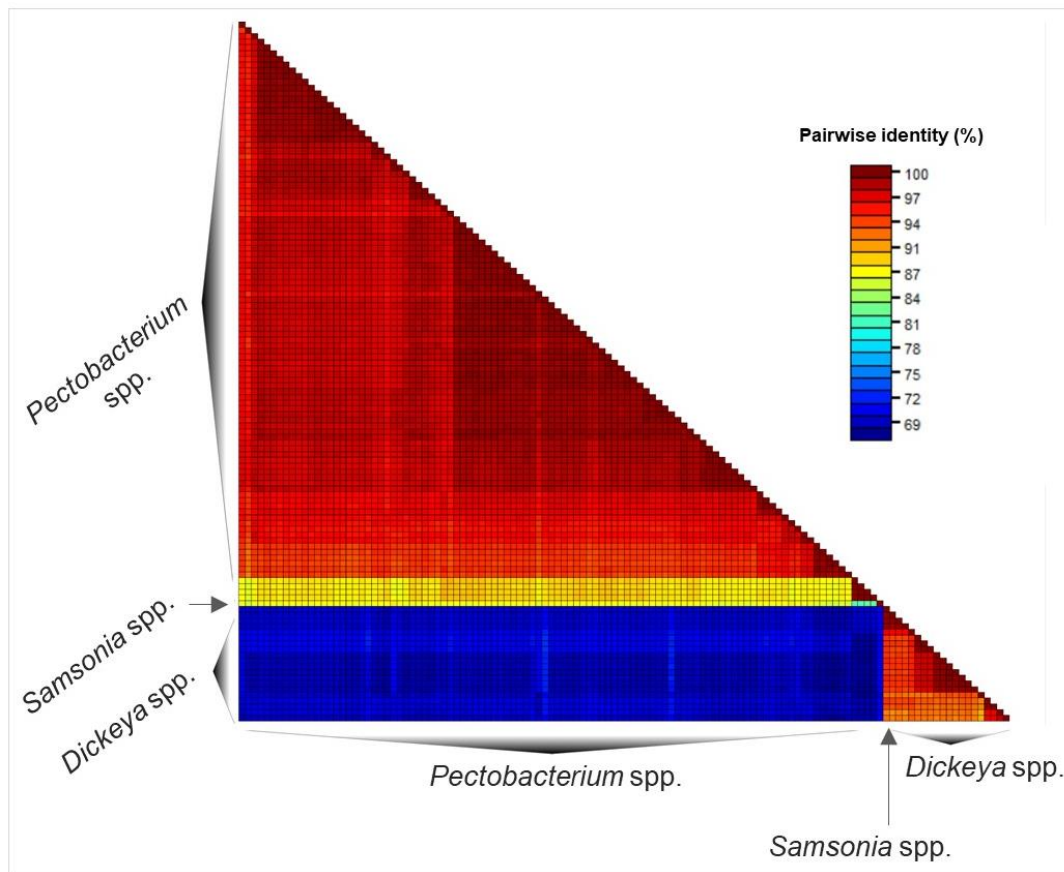


Figure 4.3: Pairwise similarity matrix and identity scores of Avrulence L homologs. The heat map shows a color-coded display of the similarity scores and percentage identities from pairwise alignments of all 122 unique AvrL proteins of *Pectobacterium*, *Samsonia*, and *Dickeya* spp. Percentage identity values increase from blue (~69%) to red (100%).

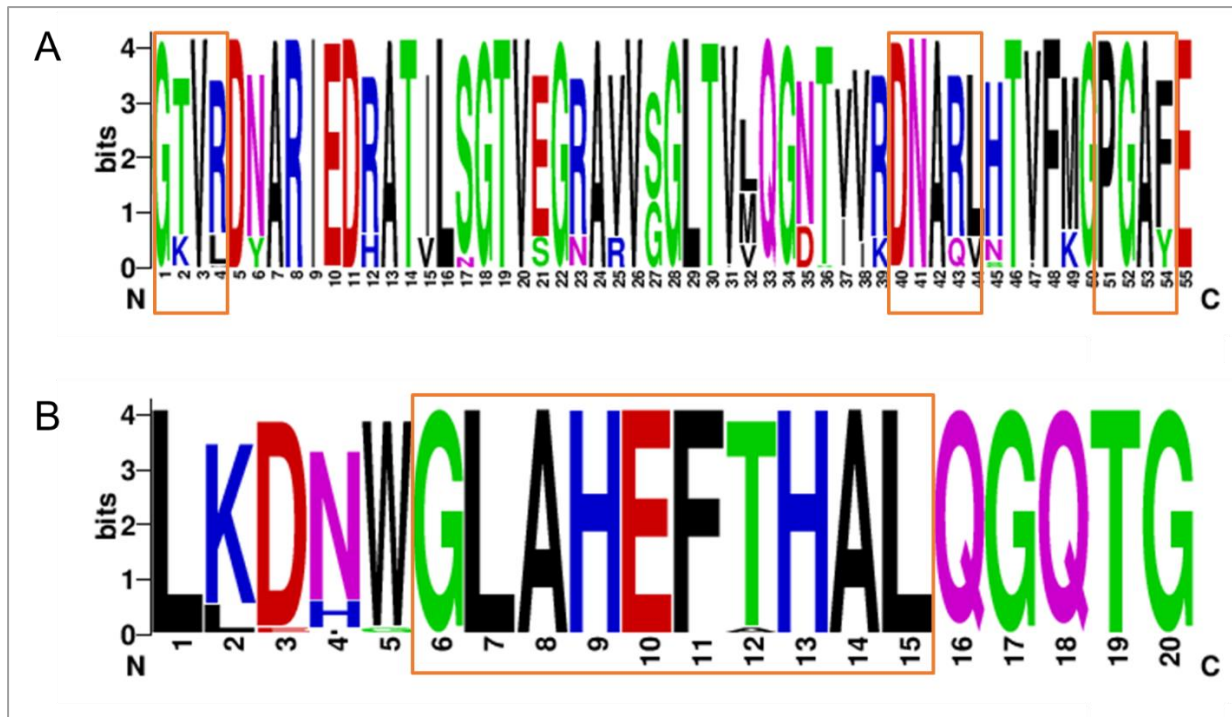


Figure 4.4: Soft Rot *Pectobacteriaceae* Avrulence L conserved domains. The image shows the WebLogo sequence alignments of SRP AvrL proteins identified with via BlastP search. (A) The logo shows the highly conserved lpxD putative domain with conserved insertions marked in boxes. (B) The logo shows the conserved zinc binding metalloproteinase domain and HEXxH motif highlighted by the orange box.

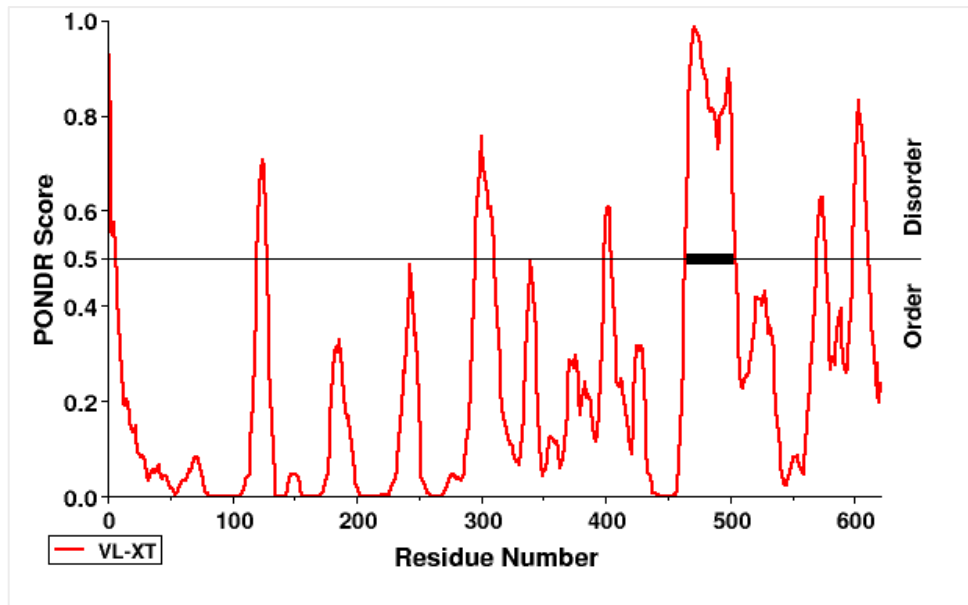


Figure 4.5: Intrinsically disordered regions (IDRs) of the *Pectobacterium brasiliense* 1692 AvrL protein. The graph shows the PONDR VL–XT disorder scores and the corresponding AvrL protein sequence residue numbers. Peaks above the 0.5 mark indicate the seven AvrL IDRs. Regions of the protein scoring below the 0.5 threshold represent ordered regions.

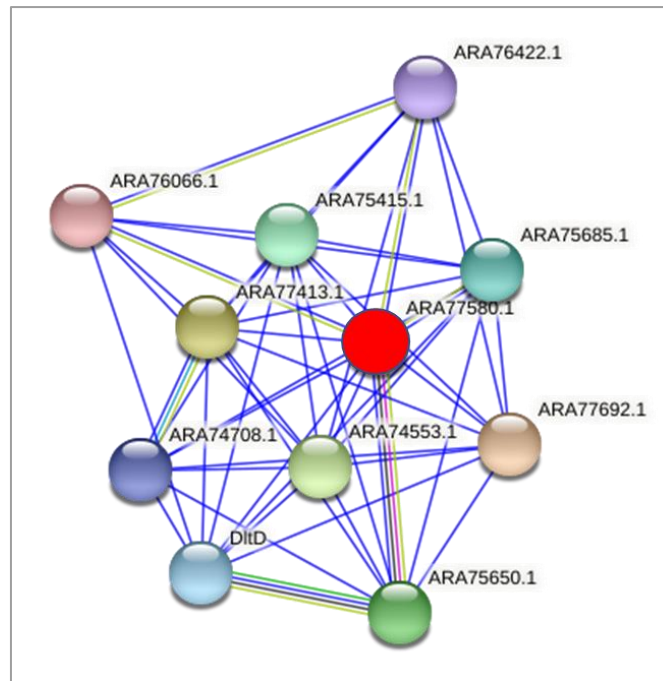


Figure 4.6: Predicted Avirulence L (AvrL) functional interactors in *Pectobacterium brasiliense* spp. The image shows the STRING predicted interactors of AvrL (red node). The other nodes represent the ten interactors rhamnogalacturonate lyase, two pectate lyases, expansin YoaJ, two uncharacterised proteins, cytochrome C550, methyltransf-25 domain containing protein, DltD, and sugar binding protein. Protein-protein interactions are shown as edges based on neighborhood (green), fusion (red), co-occurrence (blue), experiments (purple), databases (light green), text mining (yellow), and homology (light blue).

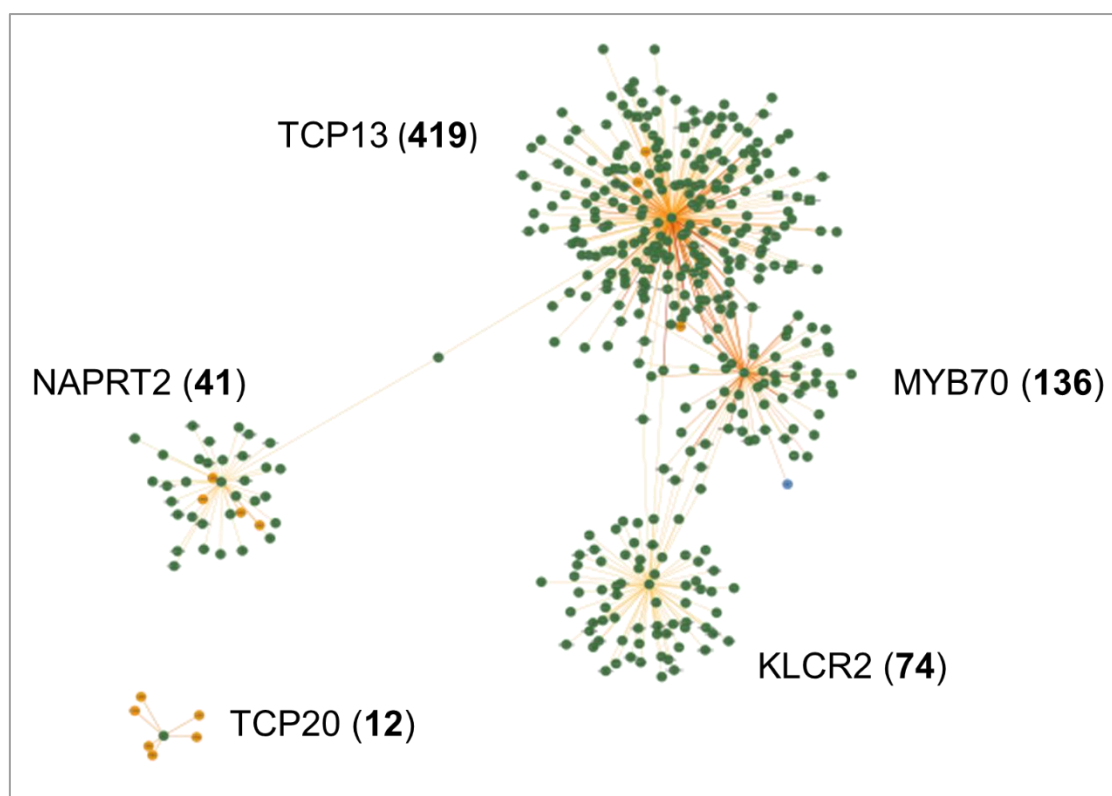


Figure 4.7: Inter-species protein-protein interactions visualised in Cytoscape. The image shows the network of the top five most probable interactors of Avirulence L built from the 152 PPI fuzzy network. Each green dot (node) represents each *Arabidopsis thaliana* protein. The lines (edges) represent the interconnections between proteins. The network shows each candidate top ranking AvrL interactor, and the number of interactions associated with it (brackets).

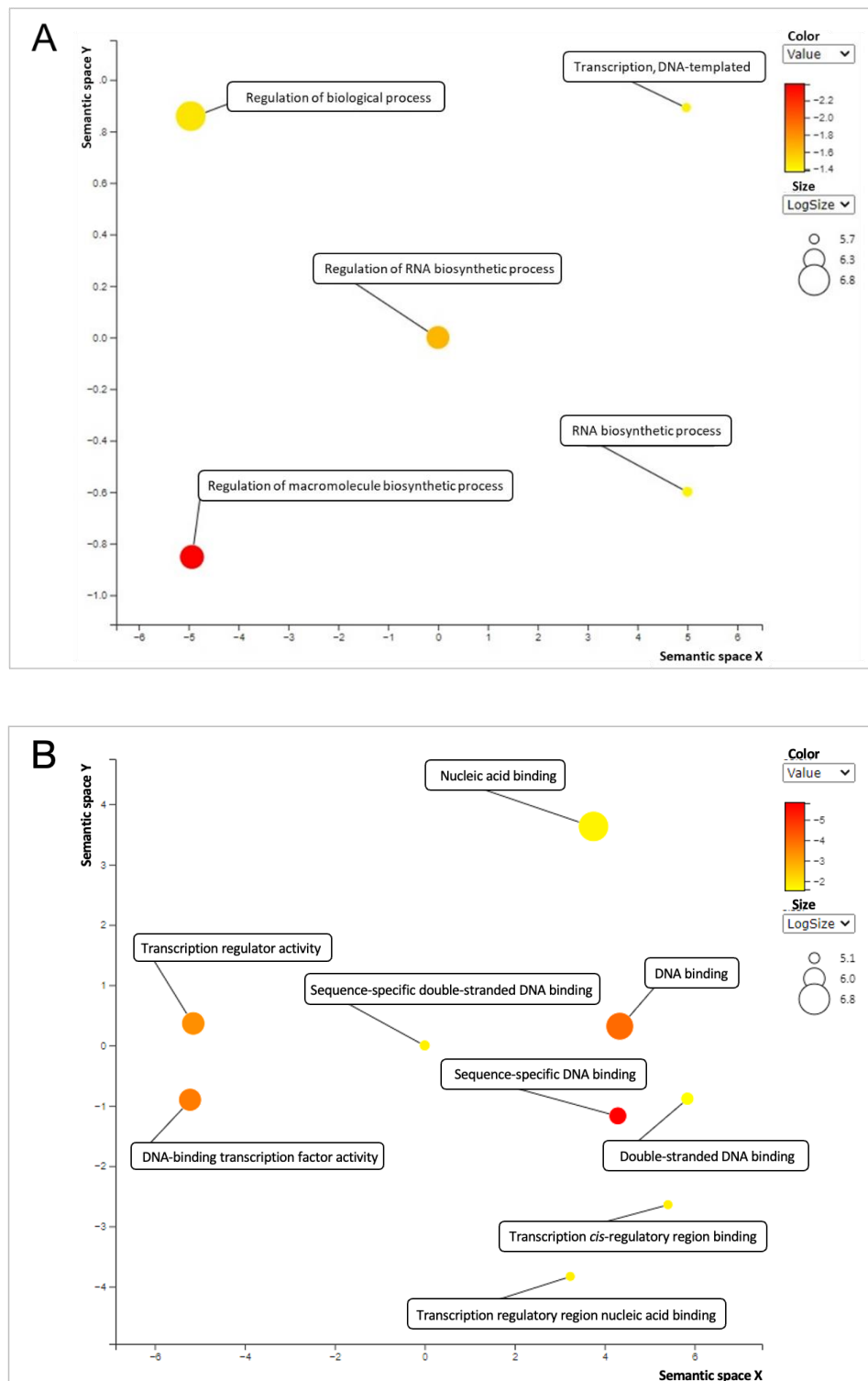


Figure 4.8: Gene ontology assignment of *Solanum tuberosum* cultivar Valor Avrulence L candidate targets. The image shows the highly enriched GO terms associated with *S. tuberosum* cv. Valor predicted to interact with AvrL. The REVIGO scatterplot shows the enriched proteins in (A) biological process ontology and (B)

molecular activity ontology. The colors indicate the p -value. The log size indicates the $\log_{10}$ (the number of proteins associated with the GO term).

Table 4.1. Webtools used in this study.

Tool name	Website	Access date	Reference
Universal Protein Resource (UniProt)	https://www.uniprot.org/	15 June 2021	UniProt, 2021
National Centre for Biotechnology Information (NCBI)	https://www.ncbi.nlm.nih.gov/	15 June 2021	Madden, 2013
Multiple Alignment using Fast Fourier Transform (MAFFT)	https://www.ebi.ac.uk/Tools/msa/mafft/	02 April 2022	Katoh & Standley, 2013
Sequence Identities and Similarities (SIAS)	http://imed.med.ucm.es/Tools/sias.html/	05 April 2022	RechP. SIAS: Sequence identities and similarities. 2008.
Sequence Demarcation Tool (SDT)	http://web.cbio.uct.ac.za/~brejnev/	06 April 2022	Muhire <i>et al.</i> , 2014
ExPASy-ProtParam tool	https://web.expasy.org/protparam/	05 June 2022	Gasteiger <i>et al.</i> , 2005
Conserved Domain Database (CDD)	https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi/	04 September 2021	Marchler-Bauer <i>et al.</i> , 2017
ExPASy-PROSITE database	https://prosite.expasy.org/index.html/	04 September 2021	Sigrist <i>et al.</i> , 2012
Predictor of Natural Disordered Regions (PONDR® VL-XT)	http://www.pondr.com/	13 August 2021	Romero <i>et al.</i> , 2004
COFACTOR	https://zhanggroup.org/COFACTOR	19 June 2022	Zhang <i>et al.</i> , 2017

STRING v11.5	https://string-db.org/	10 May 2022	Szklarczyk <i>et al.</i> , 2019
InterSPPI v3 AraPathogen tool	http://systbio.cau.edu.cn/	20 November 2021	Yang <i>et al.</i> , 2019
<i>Arabidopsis</i> Information Resource (TAIR)	https://www.arabidopsis.org/tools/	20 November 2021	Lamesch <i>et al.</i> , 2012
Database for Annotation, Visualisation, and Integrated Discovery (DAVID)	https://david.ncifcrf.gov/	13 December 2021	Huang <i>et al.</i> , 2007
REVIGO	http://revigo.irb.hr/	14 December 2021	Supek <i>et al.</i> , 2010
InterProScan	https://www.ebi.ac.uk/	08 June 2022	Jone <i>et al.</i> , 2014
g: Profiler tool	https://biit.cs.ut.ee/gprofiler/gost	08 June 2022	Raudvere <i>et al.</i> , 2019
Plant Transcription Factor Database v5.0 (PlantTFDB)	http://planttfdb.gao-lab.org/	on 30 June 2022	Zhang <i>et al.</i> , 2011

Table 4.2. Physicochemical features of *Pectobacterium brasiliense* 1692 Avirulence L protein sequence.

Seq length	MW (kDa)	pI	EC	Ii	Ai	GRAVY	-R	+R
621	68.386	7.66	147250	36.97	67.25	-0.388	55	56

Key to Table:

Seq length, sequence length

MW, Molecular weight

pI, Isoelectric point

EC, Extinction coefficients

Ii, Instability index

Ai, Aliphatic index

GRAVY, Grand average of hydropathicity

-R, Total number of negatively charged residues (Asp + Glu)

+R, Total number of positively charged residues (Arg + Lys)

Table 4.3. Potato transcription factor functional annotations.

PGSC	TAIR	Protein name	GO Term	GO Category	GO Description
PGSC0003D	AT3G61	Cutin deficient	GO:0009965	Biological	leaf morphogenesis
MG4000013	150	2		Process	
62					
			GO:0030154	Biological Process	cell differentiation
			GO:0031347	Biological Process	regulation of defence response
			GO:0045962	Biological Process	positive regulation of development, heterochronic
			GO:0009507	Cellular Component	chloroplast
PGSC0003D	AT3G02	Transcription	GO:0006355	Biological	regulation of
MG4000277	150	factor 17		Process	transcription, DNA-templated
70					
			GO:0009827	Biological Process	plant-type cell wall modification
			GO:0042335	Biological Process	cuticle development
			GO:0043481	Biological Process	anthocyanin accumulation in tissues in response to UV light
			GO:0048765	Biological Process	root hair cell differentiation
			GO:0005634	Cellular Component	nucleus

			GO:0008289	Molecular Function	lipid binding
			GO:0043565	Molecular Function	sequence-specific DNA binding
PGSC0003D MG4000013 39	AT1G18 570	Protein1	GO:0000902	Biological Process	cell morphogenesis
			GO:0009651	Biological Process	response to salt stress
			GO:0009723	Biological Process	response to ethylene
			GO:0009733	Biological Process	response to auxin
			GO:0009739	Biological Process	response to gibberellin
			GO:0009751	Biological Process	response to salicylic acid
			GO:0009753	Biological Process	response to jasmonic acid
			GO:0046686	Biological Process	response to cadmium ion
			GO:0003677	Molecular Function	DNA binding
PGSC0003D MG4000123 96	AT1G53 230	Lanceolate	GO:0009793	Biological Process	embryo development ending in seed dormancy
			GO:0009965	Biological Process	leaf morphogenesis

			GO:0030154	Biological Process	cell differentiation
			GO:0045962	Biological Process	positive regulation of development, heterochronic
			GO:0003677	Molecular Function	DNA binding
PGSC0003D MG4000023 94	AT4G17 710	Homeobox protein	GO:0006355	Biological Process	regulation of transcription, DNA-templated
			GO:0048497	Biological Process	maintenance of floral organ identity
			GO:0005634	Cellular Component	nucleus
			GO:0008289	Molecular Function	lipid binding
			GO:0043565	Molecular Function	sequence-specific DNA binding
PGSC0003D MG4000058 92	AT5G22 290	Transcription factor	GO:0009626	Biological Process	plant-type hypersensitive response
			GO:0009910	Biological Process	negative regulation of flower development
			GO:0034976	Biological Process	response to endoplasmic reticulum stress
			GO:0045893	Biological Process	positive regulation of transcription, DNA-templated

GO:0005634	Cellular Component	nucleus
GO:0005783	Cellular Component	endoplasmic reticulum
GO:0016021	Cellular Component	integral component of membrane
GO:0003677	Molecular Function	DNA binding
GO:0003700	Molecular Function	transcription factor activity, sequence- specific DNA binding

PGSC0003D MG4000000 08	AT2G45 660	Transcription factor	GO:0000060	Biological Process	protein import into nucleus, translocation
			GO:0009409	Biological Process	response to cold
			GO:0009739	Biological Process	response to gibberellin
			GO:0009911	Biological Process	positive regulation of flower development
			GO:0010077	Biological Process	maintenance of inflorescence meristem identity
			GO:0045893	Biological Process	positive regulation of transcription, DNA- templated
			GO:0005634	Cellular Component	nucleus
			GO:0005737	Cellular Component	cytoplasm

				GO:0003677	Molecular Function	DNA binding
				GO:0003700	Molecular Function	transcription factor activity, sequence- specific DNA binding
				GO:0008134	Molecular Function	transcription factor binding
				GO:0046983	Molecular Function	protein dimerisation activity
PGSC0003D MG4000336 94	AT1G14 687	Zinc homeodomain protein SZF- HD1	finger	GO:0003677	Molecular Function	DNA binding
PGSC0003D MG4000096 58	AT1G79 430	Myb transcription factor APL	family	GO:0010088	Biological Process	phloem development
				GO:0010089	Biological Process	xylem development
				GO:0045893	Biological Process	positive regulation of transcription, DNA- templated
				GO:0005634	Cellular Component	nucleus
				GO:0003677	Molecular Function	DNA binding
PGSC0003D MG4000207 16	AT2G02 540	Transcription factor		GO:0048574	Biological Process	long-day photoperiodism, flowering

GO:0003677	Molecular Function	DNA binding
GO:0042803	Molecular Function	protein homodimerisation activity

Supplementary Information

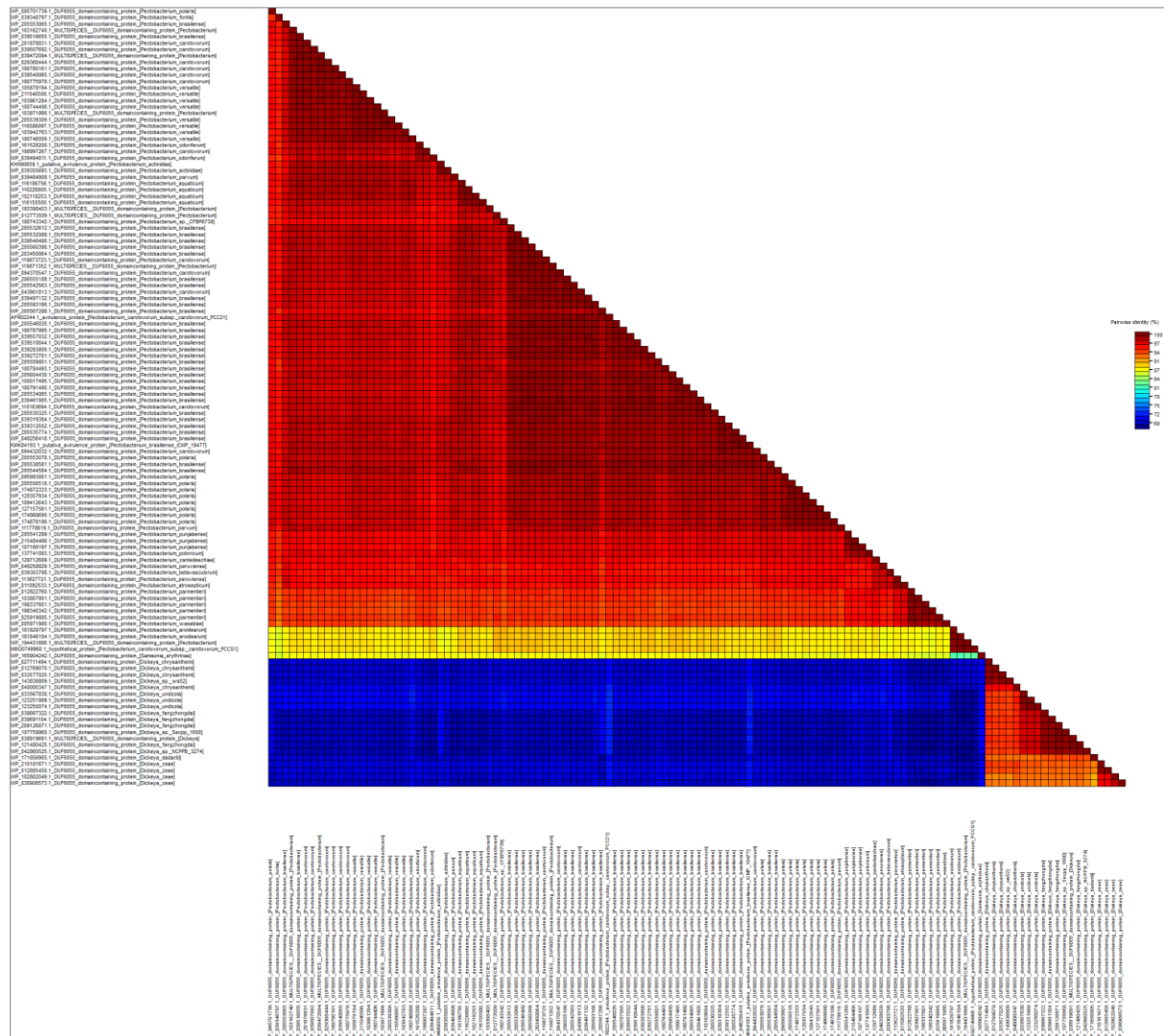


Figure S4.1: Pairwise similarity matrix of Avrulence L homologs. The matrix shows a color-coded display of the percentage identities of pairwise alignments of all unique AvrL proteins of *Pectobacterium*, *Samsonia*, and *Dickeya*. Percentage identity values increase from blue (low) -red (high).

Table S4.1. *Arabidopsis thaliana* proteins that potentially interact with *Pectobacterium brasiliense* 1692 Avirulence L protein.

Target host ID	Pathogen protein ID	Prediction score	Interaction
AT3G27010	WP_039319384	9.20E-04	Yes
AT3G02150	WP_039319384	8.80E-04	Yes
AT2G23420	WP_039319384	7.70E-04	Yes
AT2G23290	WP_039319384	7.30E-04	Yes
AT3G27960	WP_039319384	6.80E-04	Yes
AT4G39050	WP_039319384	6.70E-04	Yes
AT3G17860	WP_039319384	5.50E-04	Yes
AT2G45680	WP_039319384	5.20E-04	Yes
AT4G25200	WP_039319384	5.00E-04	Yes
AT1G54060	WP_039319384	4.90E-04	Yes
AT5G22310	WP_039319384	4.90E-04	Yes
AT3G47620	WP_039319384	4.70E-04	Yes
AT4G02150	WP_039319384	4.60E-04	Yes
AT4G19700	WP_039319384	4.00E-04	Yes
AT4G08150	WP_039319384	3.90E-04	Yes
AT3G08530	WP_039319384	3.90E-04	Yes
AT1G25490	WP_039319384	3.80E-04	Yes
AT1G14340	WP_039319384	3.60E-04	Yes
AT5G06780	WP_039319384	3.50E-04	Yes
AT4G17680	WP_039319384	3.30E-04	Yes
AT1G22920	WP_039319384	3.20E-04	Yes
AT2G41350	WP_039319384	3.20E-04	Yes
AT3G07780	WP_039319384	3.20E-04	Yes
AT4G26110	WP_039319384	3.00E-04	Yes
AT4G35580	WP_039319384	3.00E-04	Yes
AT5G51910	WP_039319384	3.00E-04	Yes
AT2G44950	WP_039319384	2.90E-04	Yes
AT5G24660	WP_039319384	2.90E-04	Yes
AT1G06510	WP_039319384	2.80E-04	Yes
AT4G16143	WP_039319384	2.60E-04	Yes
AT4G02590	WP_039319384	2.40E-04	Yes
AT5G56950	WP_039319384	2.30E-04	Yes
AT5G58720	WP_039319384	2.10E-04	Yes
AT2G02540	WP_039319384	2.10E-04	Yes
AT3G49580	WP_039319384	2.10E-04	Yes
AT4G01090	WP_039319384	1.90E-04	Yes
AT4G24840	WP_039319384	1.90E-04	Yes

AT3G13720	WP_039319384	1.80E-04	Yes
AT5G55100	WP_039319384	1.80E-04	Yes
AT1G15750	WP_039319384	1.70E-04	Yes
AT4G28640	WP_039319384	1.70E-04	Yes
AT3G11590	WP_039319384	1.60E-04	Yes
AT4G32570	WP_039319384	1.50E-04	Yes
AT1G69690	WP_039319384	1.40E-04	Yes
AT5G02150	WP_039319384	1.40E-04	Yes
AT5G60120	WP_039319384	1.30E-04	Yes
AT4G11890	WP_039319384	1.10E-04	Yes
AT3G53410	WP_039319384	9.90E-05	Yes
AT4G25660	WP_039319384	9.70E-05	Yes
AT1G21200	WP_039319384	9.60E-05	Yes
AT3G56270	WP_039319384	9.50E-05	Yes
AT3G09630	WP_039319384	9.40E-05	Yes
AT5G19990	WP_039319384	9.30E-05	Yes
AT5G61010	WP_039319384	9.20E-05	Yes
AT4G15545	WP_039319384	9.10E-05	Yes
AT3G06720	WP_039319384	9.10E-05	Yes
AT3G51960	WP_039319384	9.00E-05	Yes
AT3G01500	WP_039319384	8.70E-05	Yes
AT5G52750	WP_039319384	8.60E-05	Yes
AT3G57870	WP_039319384	8.50E-05	Yes
AT3G10480	WP_039319384	8.40E-05	Yes
AT1G18570	WP_039319384	8.40E-05	Yes
AT3G61150	WP_039319384	8.30E-05	Yes
AT3G17410	WP_039319384	8.20E-05	Yes
AT4G36970	WP_039319384	8.10E-05	Yes
AT4G26450	WP_039319384	8.00E-05	Yes
AT3G48150	WP_039319384	8.00E-05	Yes
AT3G61790	WP_039319384	7.90E-05	Yes
AT4G36540	WP_039319384	7.90E-05	Yes
AT5G19480	WP_039319384	7.70E-05	Yes
AT3G49570	WP_039319384	7.60E-05	Yes
AT1G71230	WP_039319384	7.40E-05	Yes
AT4G15730	WP_039319384	7.30E-05	Yes
AT4G25920	WP_039319384	7.10E-05	Yes
AT3G54850	WP_039319384	7.00E-05	Yes
AT1G55190	WP_039319384	6.80E-05	Yes
AT1G17970	WP_039319384	6.30E-05	Yes

AT1G75080	WP_039319384	6.30E-05	Yes
AT1G21700	WP_039319384	6.20E-05	Yes
AT5G22355	WP_039319384	6.10E-05	Yes
AT5G56290	WP_039319384	6.00E-05	Yes
AT4G30080	WP_039319384	6.00E-05	Yes
AT5G59650	WP_039319384	5.90E-05	Yes
AT3G21490	WP_039319384	5.90E-05	Yes
AT1G53230	WP_039319384	5.90E-05	Yes
AT1G45145	WP_039319384	5.60E-05	Yes
AT2G21380	WP_039319384	5.40E-05	Yes
AT2G39760	WP_039319384	5.40E-05	Yes
AT2G01760	WP_039319384	5.40E-05	Yes
AT1G27300	WP_039319384	5.20E-05	Yes
AT5G27860	WP_039319384	5.10E-05	Yes
AT1G76850	WP_039319384	5.00E-05	Yes
AT2G45660	WP_039319384	5.00E-05	Yes
AT2G02100	WP_039319384	4.90E-05	Yes
AT1G02690	WP_039319384	4.90E-05	Yes
AT4G17710	WP_039319384	4.80E-05	Yes
AT2G43370	WP_039319384	4.60E-05	Yes
AT1G63480	WP_039319384	4.60E-05	Yes
AT1G32640	WP_039319384	4.40E-05	Yes
AT5G02050	WP_039319384	4.30E-05	Yes
AT4G26840	WP_039319384	4.20E-05	Yes
AT3G48550	WP_039319384	4.10E-05	Yes
AT2G47060	WP_039319384	4.10E-05	Yes
AT1G10585	WP_039319384	4.00E-05	Yes
AT1G18400	WP_039319384	4.00E-05	Yes
AT2G25000	WP_039319384	3.90E-05	Yes
AT4G34460	WP_039319384	3.80E-05	Yes
AT3G09980	WP_039319384	3.80E-05	Yes
AT3G16310	WP_039319384	3.80E-05	Yes
AT4G04020	WP_039319384	3.70E-05	Yes
AT3G25070	WP_039319384	3.70E-05	Yes
AT5G56250	WP_039319384	3.70E-05	Yes
AT3G22960	WP_039319384	3.70E-05	Yes
AT3G53350	WP_039319384	3.60E-05	Yes
AT4G22240	WP_039319384	3.50E-05	Yes
AT5G66560	WP_039319384	3.40E-05	Yes
AT1G14687	WP_039319384	3.40E-05	Yes

AT3G54170	WP_039319384	3.30E-05	Yes
AT5G42480	WP_039319384	3.10E-05	Yes
AT4G17220	WP_039319384	3.10E-05	Yes
AT5G22290	WP_039319384	3.00E-05	Yes
AT2G46020	WP_039319384	3.00E-05	Yes
AT3G10250	WP_039319384	3.00E-05	Yes
AT3G54390	WP_039319384	2.90E-05	Yes
AT2G25650	WP_039319384	2.90E-05	Yes
AT3G18130	WP_039319384	2.80E-05	Yes
AT4G37130	WP_039319384	2.60E-05	Yes
AT5G20030	WP_039319384	2.50E-05	Yes
AT1G75950	WP_039319384	2.40E-05	Yes
AT5G08330	WP_039319384	2.30E-05	Yes
AT3G63210	WP_039319384	2.30E-05	Yes
AT1G58100	WP_039319384	2.20E-05	Yes
AT1G12520	WP_039319384	2.20E-05	Yes
AT5G42980	WP_039319384	2.10E-05	Yes
AT2G35940	WP_039319384	2.00E-05	Yes
AT5G65210	WP_039319384	2.00E-05	Yes
AT1G14920	WP_039319384	2.00E-05	Yes
AT2G01570	WP_039319384	1.80E-05	Yes
AT2G15960	WP_039319384	1.60E-05	Yes
AT2G34710	WP_039319384	1.50E-05	Yes
AT1G29990	WP_039319384	1.30E-05	Yes
AT5G11980	WP_039319384	1.30E-05	Yes
AT1G09270	WP_039319384	1.30E-05	Yes
AT1G30860	WP_039319384	1.20E-05	Yes
AT5G13810	WP_039319384	9.00E-06	Yes
AT1G79430	WP_039319384	9.00E-06	Yes
AT5G66480	WP_039319384	9.00E-06	Yes
AT1G23380	WP_039319384	8.00E-06	Yes
AT5G57210	WP_039319384	8.00E-06	Yes
AT3G01670	WP_039319384	3.00E-06	Yes
AT5G59670	WP_039319384	1.00E-06	Yes
AT4G32190	WP_039319384	0.00E+00	Yes

Table S4.2. Avirulence L top ranking candidate *Arabidopsis thaliana* interactors.

Target host proteins	Gene name
AT3G27010	TEOSINTE BRANCHED 1, cycloidea, PCF (TCP)-domain family protein 20(TCP20)
AT3G02150	Plastid transcription factor 1(PTF1) (TCP13)
AT2G23420	nicotinate phosphoribosyltransferase 2(NAPRT2)
AT2G23290	Myb domain protein 70(MYB70)
AT3G27960	Tetratricopeptide repeat (TPR)-like superfamily protein (KLCR2)
AT4G39050	Kinesin motor family protein (AT4G39050)
AT3G17860	Jasmonate-zim-domain protein 3(JAZ3)
AT2G45680	TCP family transcription factor (TCP9)
AT4G25200	mitochondrion-localised small heat shock protein 23
AT1G54060	6B-interacting protein 1-like 1(ASIL1)
AT5G22310	trichohyalin-like protein (AT5G22310)
AT3G47620	TEOSINTE BRANCHED, cycloidea and PCF (TCP) 14(TCP14)
AT4G02150	ARM repeat superfamily protein (MOS6)
AT4G19700	SBP (S-ribonuclease binding protein) family protein (RING)
AT4G08150	homeobox knotted-like protein (KNAT1)
AT3G08530	Clathrin, heavy chain (AT3G08530)
AT1G25490	Kinesin motor family protein (AT4G39050)
AT1G14340	RNA-binding (RRM/RBD/RNP motifs) family protein (AT1G14340)
AT5G06780	Emsy N Terminus (ENT)/ plant Tudor-like domains-containing protein (EML2)
AT4G17680	SBP (S-ribonuclease binding protein) family protein (AT4G17680)

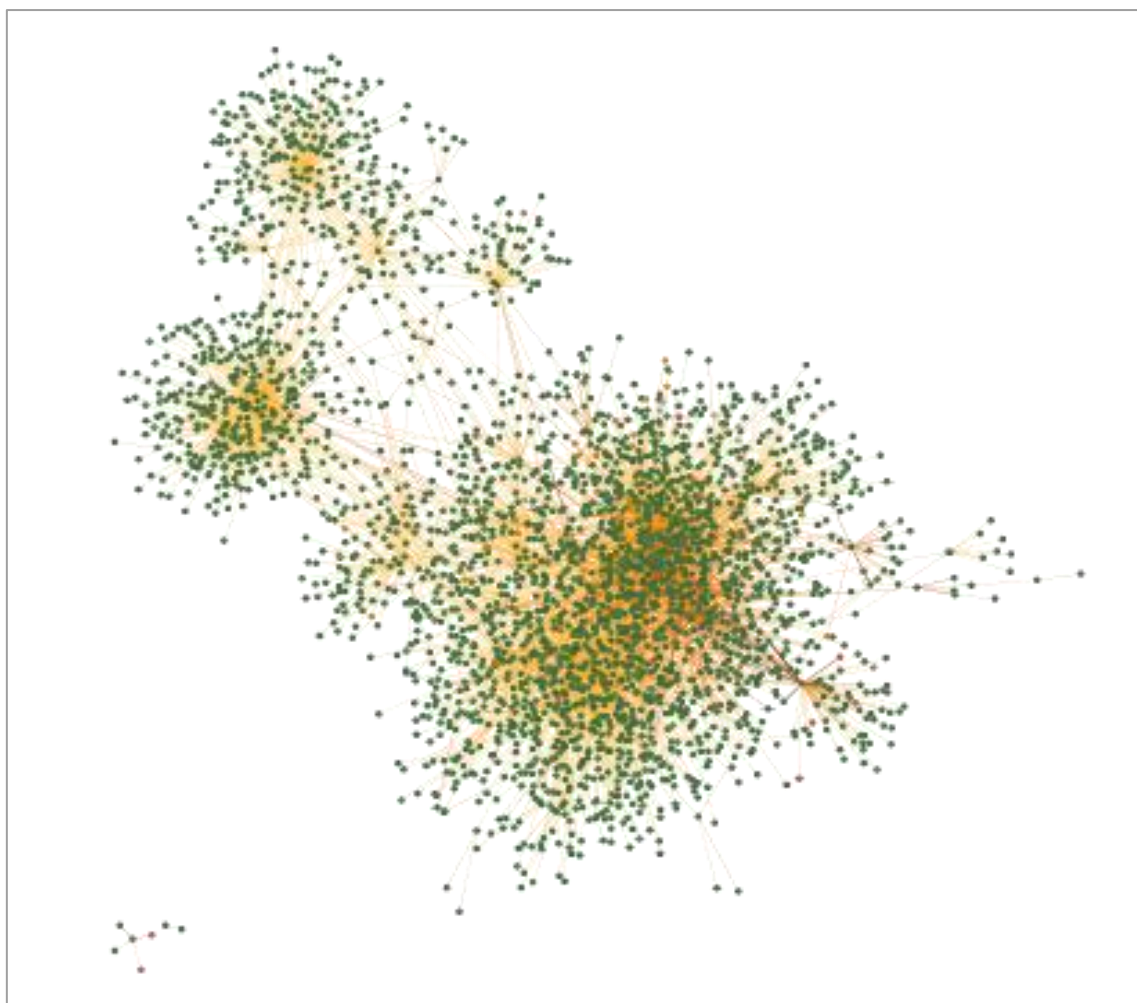
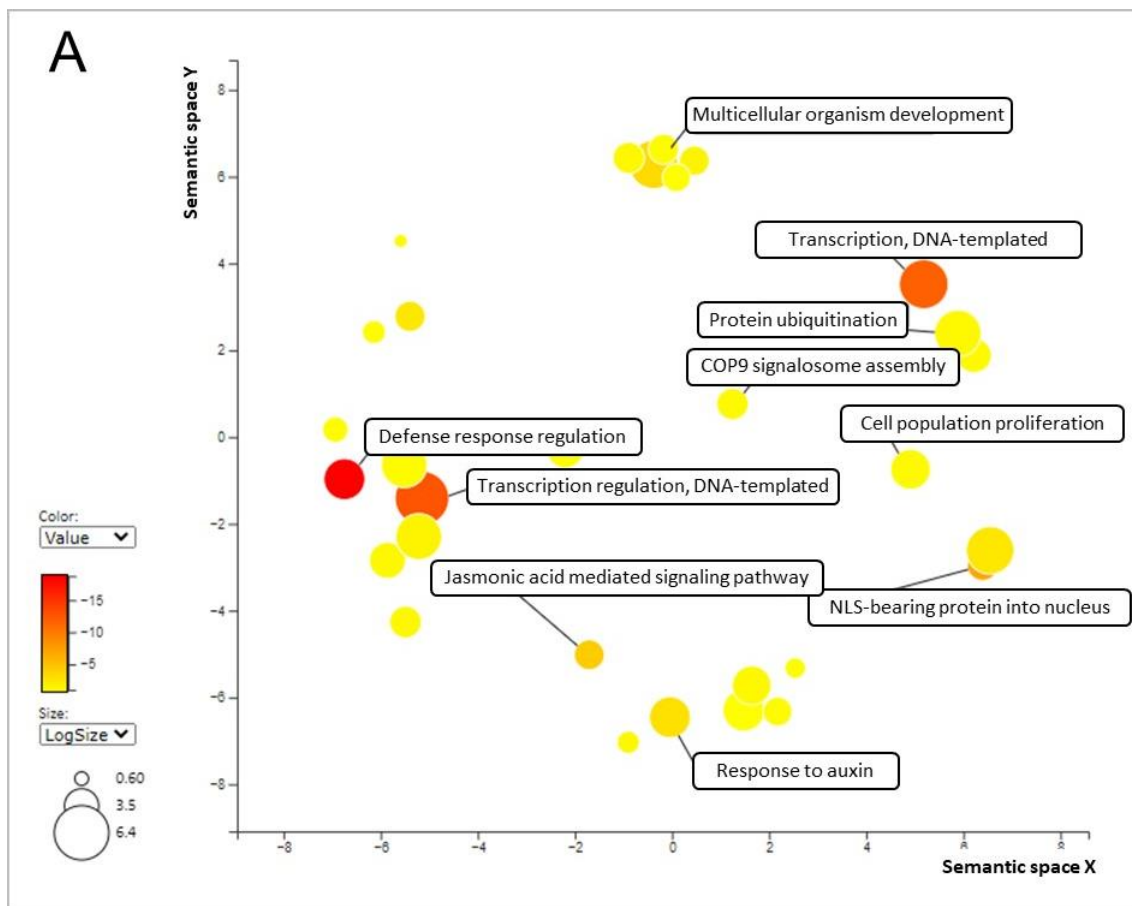
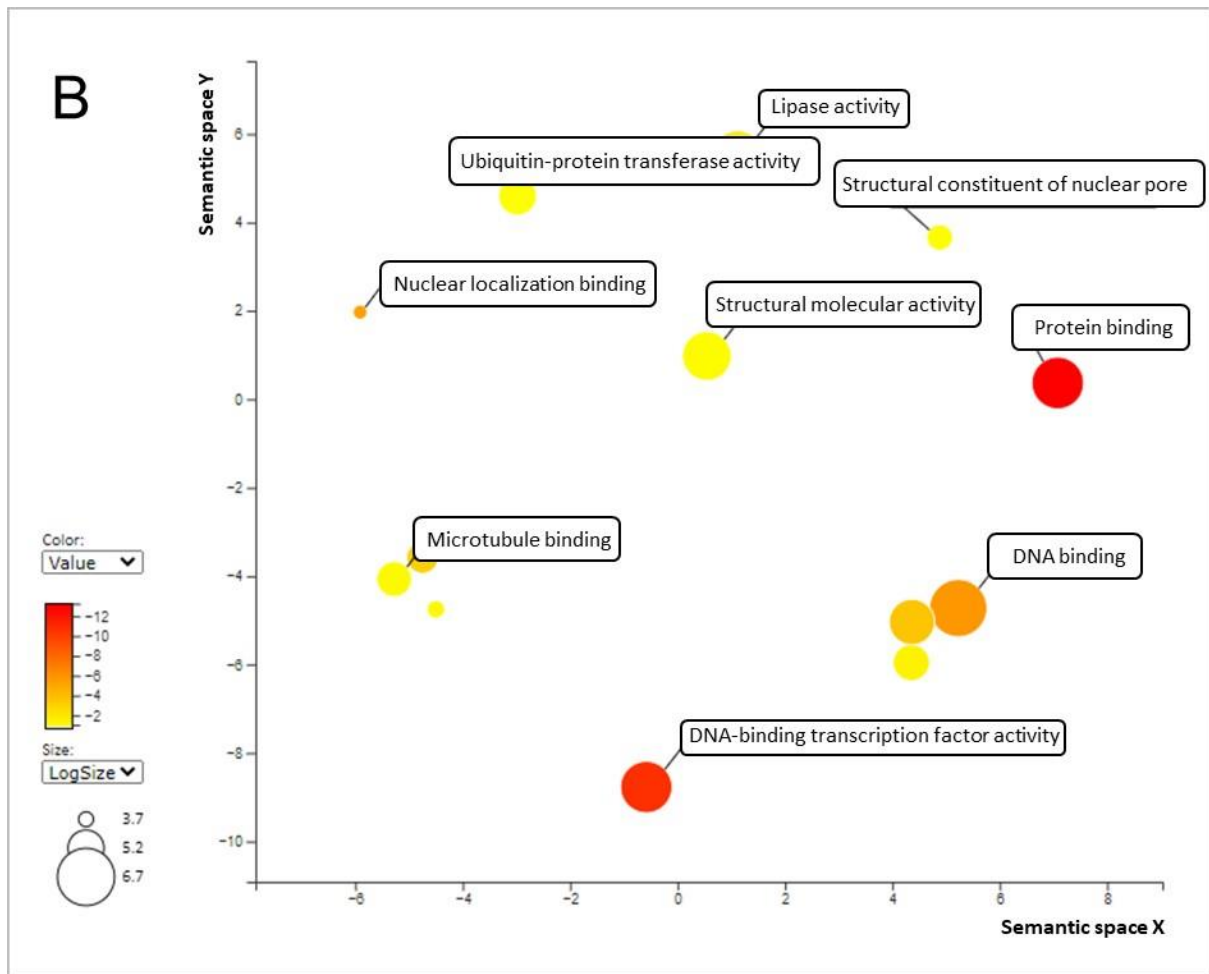


Figure S4.2: Inter-species protein-protein interaction. The fuzzy Cytoscape IntAct network shows the entire InterSPPI PPI network of interactors of AvrL. Each green dot represents each *Arabidopsis thaliana* protein. Other colors represent interactors from other organisms.





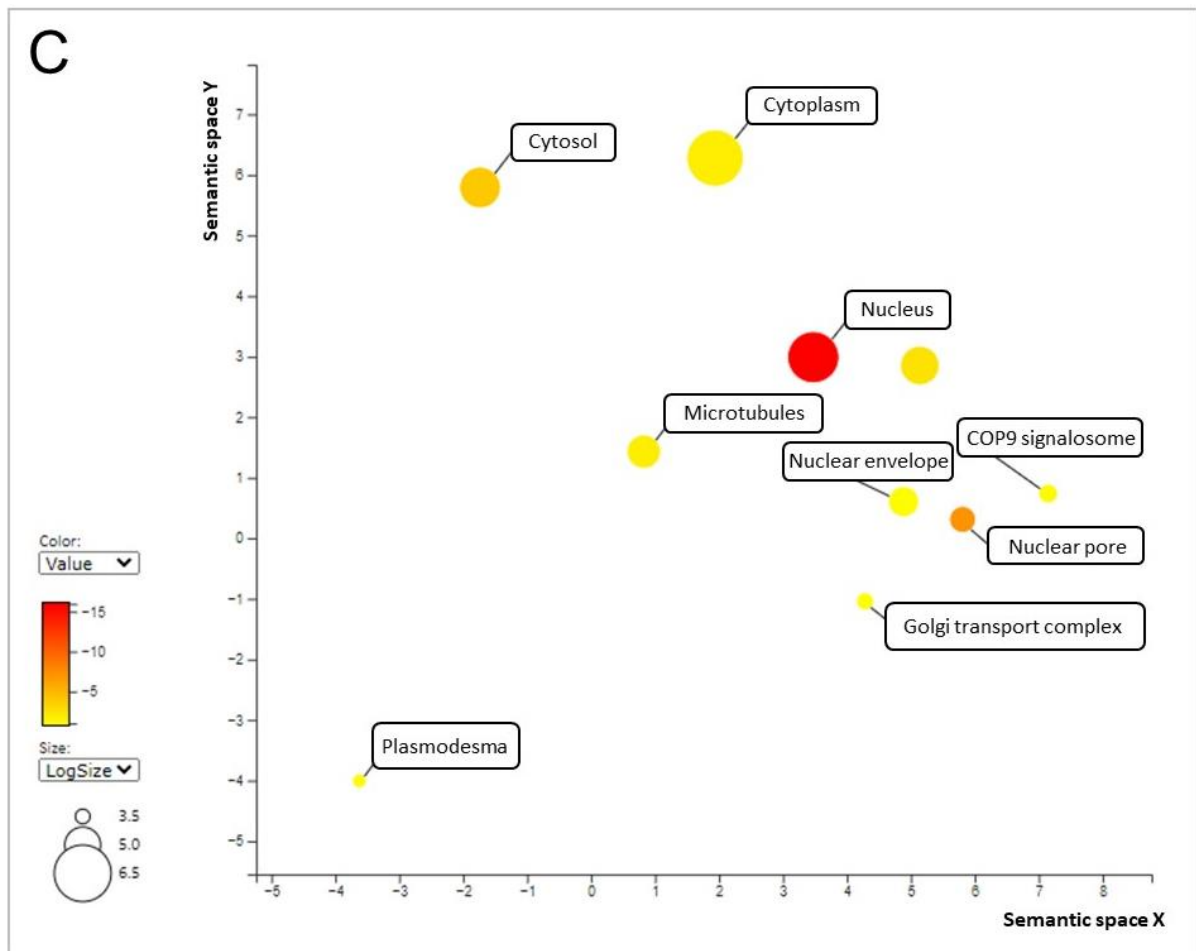


Figure S4.3: Functional annotation of candidate Avrulence L interacting proteins in *Arabidopsis thaliana*. The image shows the highly enriched GO terms associated with *A. thaliana* proteins predicted to interact with AvrL. The REVIGO scatterplot shows the enriched proteins in (A) biological process ontology, (B) molecular activity ontology, and (C) cellular component ontology. The colors indicate the p -value. The log size indicates the $\log_{10}$ (the number of proteins associated with the GO term).

Table S4.3. Functional enrichment of potato *Arabidopsis thaliana* homologs.

Ontology	Term name	GO term	Adjusted <i>p</i> -value	Number of proteins
GO:MF	sequence-specific DNA binding	GO:0043565	1.77E-13	32
GO:MF	DNA-binding transcription factor activity	GO:0003700	5.10E-11	32
GO:MF	transcription regulator activity	GO:0140110	1.20E-10	33
GO:MF	DNA binding	GO:0003677	1.40E-09	40
GO:MF	transcription regulatory region nucleic acid binding	GO:0001067	9.12E-06	18
GO:MF	transcription cis-regulatory region binding	GO:0000976	9.12E-06	18
GO:MF	sequence-specific double-stranded DNA binding	GO:1990837	1.21E-05	18
GO:MF	double-stranded DNA binding	GO:0003690	6.6E-05	18
GO:MF	nucleic acid binding	GO:0003676	0.000337	44
GO:MF	binding	GO:0005488	0.000445	94
GO:MF	DNA-binding transcription factor activity, RNA polymerase II-specific	GO:0000981	0.002749	10
GO:MF	nuclear localisation sequence binding	GO:0008139	0.019458	3
GO:MF	signal sequence binding	GO:0005048	0.020663	4
GO:MF	deNEDDylase activity	GO:0019784	0.024995	2
GO:MF	identical protein binding	GO:0042802	0.046833	5
GO:MF	protein homodimerisation activity	GO:0042803	0.048895	4
GO:BP	regulation of RNA metabolic process	GO:0051252	3.68E-10	44
GO:BP	regulation of transcription, DNA-templated	GO:0006355	5.32E-10	43
GO:BP	regulation of RNA biosynthetic process	GO:2001141	5.32E-10	43
GO:BP	regulation of nucleic acid-templated transcription	GO:1903506	5.32E-10	43
GO:BP	regulation of macromolecule biosynthetic process	GO:0010556	1.05E-09	44
GO:BP	regulation of nucleobase-containing compound metabolic process	GO:0019219	1.39E-09	44
GO:BP	regulation of cellular biosynthetic process	GO:0031326	1.47E-09	44
GO:BP	regulation of biosynthetic process	GO:0009889	1.62E-09	44
GO:BP	regulation of primary metabolic process	GO:0080090	3.22E-09	48
GO:BP	regulation of cellular metabolic process	GO:0031323	4.56E-09	48
GO:BP	transcription, DNA-templated	GO:0006351	5.19E-09	43
GO:BP	regulation of biological process	GO:0050789	5.77E-09	61
GO:BP	nucleic acid-templated transcription	GO:0097659	5.85E-09	43
GO:BP	RNA biosynthetic process	GO:0032774	6.11E-09	43

GO:BP	regulation of cellular process	GO:0050794	6.81E-09	58
GO:BP	regulation of nitrogen compound metabolic process	GO:0051171	9.75E-09	47
GO:BP	regulation of gene expression	GO:0010468	2.15E-08	44
GO:BP	regulation of macromolecule metabolic process	GO:0060255	3.20E-08	48
GO:BP	nucleobase-containing compound biosynthetic process	GO:0034654	5.28E-08	44
GO:BP	regulation of metabolic process	GO:0019222	8.42E-08	48
GO:BP	biological regulation	GO:0065007	2.25E-07	63
GO:BP	heterocycle biosynthetic process	GO:0018130	1.44E-06	44
GO:BP	aromatic compound biosynthetic process	GO:0019438	3.6E-06	44
GO:BP	organic cyclic compound biosynthetic process	GO:1901362	9.2E-06	44
GO:BP	macromolecule biosynthetic process	GO:0009059	3.88E-05	46
GO:BP	cellular nitrogen compound biosynthetic process	GO:0044271	4.22E-05	46
GO:BP	RNA metabolic process	GO:0016070	4.47E-05	46
GO:BP	nucleic acid metabolic process	GO:0090304	0.000451	48
GO:BP	gene expression	GO:0010467	0.000528	48
GO:BP	nucleobase-containing compound metabolic process	GO:0006139	0.001251	50
GO:BP	NLS-bearing protein import into nucleus	GO:0006607	0.005844	3
GO:BP	heterocycle metabolic process	GO:0046483	0.012374	50
GO:BP	cellular aromatic compound metabolic process	GO:0006725	0.039954	50
GO:CC	nucleus	GO:0005634	1.67E-12	64
GO:CC	intracellular anatomical structure	GO:0005622	1.70E-07	90
GO:CC	intracellular organelle	GO:0043229	2.89E-06	80
GO:CC	organelle	GO:0043226	3.11E-06	80
GO:CC	intracellular membrane-bounded organelle	GO:0043231	5.99E-06	76
GO:CC	membrane-bounded organelle	GO:0043227	6.7E-06	76
GO:CC	vesicle tethering complex	GO:0099023	0.007365	5
KEGG	Nucleocytoplasmic transport	KEGG:03013	0.003828	4

Key to Table:

GO:MF, Gene ontology: Molecular function

GO:BP, Gene ontology: Biological processes

GO:CC, Gene ontology: Cellular component

KEGG, Kyoto Encyclopaedia of Genes and Genomes

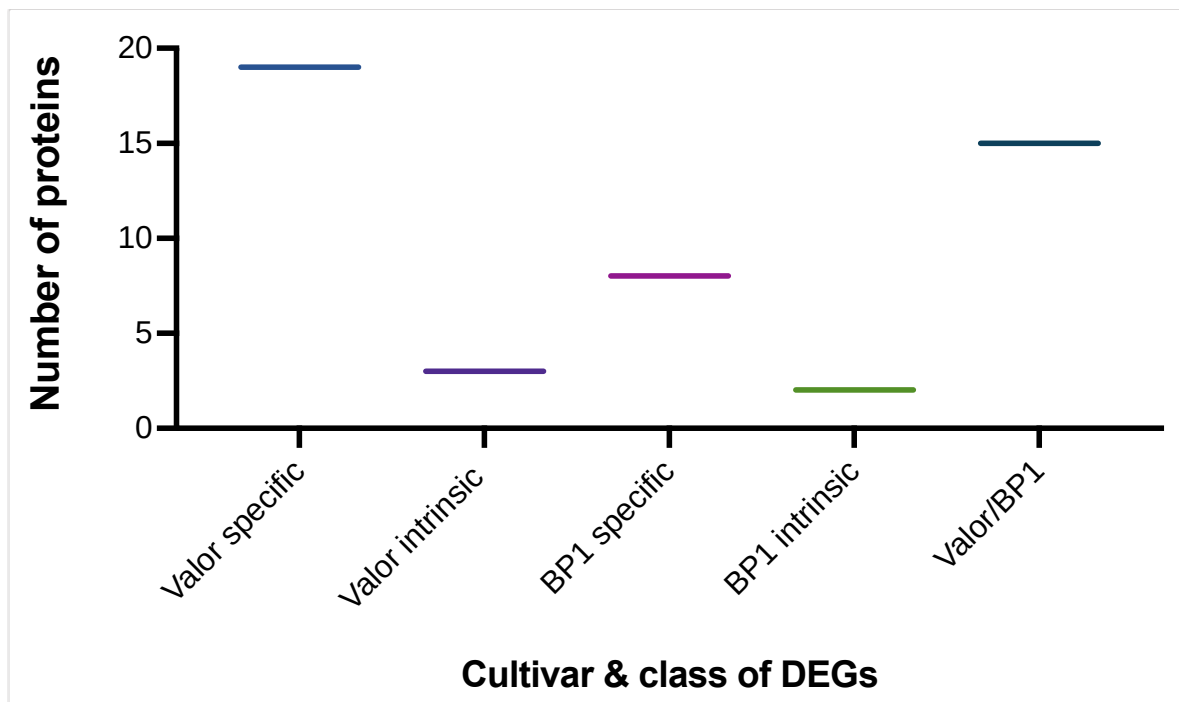


Figure S4.4: Differentially expressed predicted Avrulence L target genes in *Solanum tuberosum* cultivars infected with *Pectobacterium brasiliense* 1692. The graph shows the number of expressed genes by tolerant and susceptible potato cultivars *S. tuberosum* cv. BP1 and Valor respectively. A total of 15 proteins are expressed by both cultivars. *S. tuberosum* cv. Valor expresses 19 potential AvrL targets, three of which are intrinsic to the cultivar. *S. tuberosum* cv. BP1 expresses eight potential targets of AvrL. Two BP1 proteins are intrinsic to BP1.

Table S4.4. Avirulence L protein targets expressed by cultivar *Solunum tuberosum* cv. Valor and *S. tuberosum* cv. BP1.

			Log2 Fold Changes			
Gene ID	Araport ID	Description	6 hpi	12 hpi	24 hpi	72 hpi
Valor specific genes						
PGSC0003DMG400027770	AT3G61150	Cutin deficient 2	-		-1.21	-1.35
PGSC0003DMG400001339	AT1G18570	Protein 1	-		-	-3.72
PGSC0003DMG400012396	AT1G53230	Lanceolate	-	-3.07	-	-1.23
PGSC0003DMG400002394	AT4G17710	Homeobox protein	-	-3.59	-	-2.95
PGSC0003DMG400005892	AT5G22290	Transcription factor	-	-1.11	1.12	-
PGSC0003DMG400000523	AT3G27960	Kinesin light chain	-2.27	-3.33	-	-
PGSC0003DMG400015156	AT3G01670	Conserved gene of unknown function	-4.90	1.16	-	-
PGSC0003DMG400000008	AT2G45660	Transcription factor	-1.25	-1.69	-	-
PGSC0003DMG400026479	AT3G21490	Metal ion binding protein	-1.93	-	-	-
PGSC0003DMG400010789	AT1G76850	Exocyst complex component	-1.43	-	-	-
PGSC0003DMG400032251	AT3G63210	Conserved gene of unknown function	-	-	-	-
PGSC0003DMG400004306	AT4G32190	Ubiquitin-protein ligase BRE1A	-	-	-	-
PGSC0003DMG400033694	AT1G14687	Zinc finger homeodomain protein SZF-HD1	-	1.92	-	-
PGSC0003DMG400009658	AT1G79430	Myb family transcription factor APL	-	-1.36	-	-1.15
PGSC0003DMG400016727	AT4G22240	Light-induced protein, chloroplastic	-	-5.34	-	1.41
PGSC0003DMG400024368	AT4G19700	Conserved gene of unknown function	-	-	-	1.17
PGSC0003DMG400020716	AT2G02540	Transcription factor	-	-	-	-2.73
PGSC0003DMG400001362	AT3G02150	Transcription factor	-	-	-	-1.10
PGSC0003DMG400018105	AT4G25920	Ubiquitin-protein ligase	-	-	-	-6.46
BP1 specific genes						

PGSC0003DMG400025390	AT5G60120	Apetala 2	-		-1.33	-1.04
PGSC0003DMG400004012	AT2G23420	Nicotinate phosphoribosyltransferase	-		1.42	-
PGSC0003DMG400001498	AT4G28640	Auxin-responsive protein IAA13	-	-1.63	-	-
PGSC0003DMG400005408	AT3G54390	Transcription factor	-	1.70	-	-
PGSC0003DMG401000722	AT4G34460	Guanine nucleotide-binding protein subunit beta	-	-1.36	-	-
PGSC0003DMG400006481	AT3G57870	Ubiquitin carrier protein	-	-1.33	-	-
PGSC0003DMG400026574	AT3G56270	Myosin heavy chain	-	1.14	-	1.23
PGSC0003DMG400005891	AT5G22310	Conserved gene of unknown function	-	1.012	-	1.18
Valor and BP1 common genes						
PGSC0003DMG400019824	AT2G25000	JA-induced WRKY protein	2.22 / 3.21	0 / 3.82	0 / 3.19	2.67 / 0
PGSC0003DMG400006321	AT5G22355	Conserved gene of unknown function	12.19 / 0	12.13 / 3.99	11.39 / 3.35	10.32 / 0
PGSC0003DMG400017592	AT3G53350	P70 protein	-1.05 / 0	-1.11 / -1.19	0 / 0	0 / 0
PGSC0003DMG400004808	AT4G25200	Mitochondrial small heat shock protein	-1.23 / -0.76	-0.95 / -1.05	1.21 / 2.16	2.72 / 3.36
PGSC0003DMG400005388	AT1G18400	Basic helix-loop-helix protein BHLH5	0 / 0	0 / 1.46	-1.31 / 0	0 / 0
PGSC0003DMG400015482	AT4G36540	Transcription factor bHLH63	-1.69 / 0	0 / -1.39	-1.35 / -1.56	-1.99 / -1.14
PGSC0003DMG400018091	AT3G48550	Nucleic acid binding protein	0 / 0	0 / -0.84	0 / -1.52	-1.77 / -1.30
PGSC0003DMG400010034	AT5G66560	Photoreceptor-interacting protein	-1.10 / 0	0 / 0	0 / -1.35	0 / -0.87
PGSC0003DMG400000546	AT3G49570	UP-9A	-1.29 / -1.42	-1.33 / 0	0 / 0	1.86 / 1.55
PGSC0003DMG400026345	AT5G22310	Conserved gene of unknown function	-0.66 /	0 / 0	0 / 0	-1.05 / -1.18
PGSC0	AT5g02050	Conserved gene of unknown function	1.59 / 0	0 / 0	0 / 0	0 / -1.21

003DMG400021701						
PGSC0003DMG400034312	AT1g27300	Conserved gene of unknown function	-1.11 / 0	0 / 0	1.38 / 0	2.60 / 1.38
PGSC0003DMG400000545	AT5G24660	UP-9A	-1.26 / 0	-2.32 / 0	0 / 0	1.10 / 1.29
PGSC0003DMG400000493	AT3G01500	Carbonic anhydrase	-6.60	0 / -5.24	-3.02 / 0	-6.29 / -3.11
PGSC0003DMG400020510	AT4G17680	Ubiquitin-protein ligase	0 / -1.87	0 / -2.28	0 / -1.28	-1.22 / 0

Key to table

^aFold change values for *S. tuberosum* cv. Valor and BP1 per timepoint are separated by “/”.

CHAPTER FIVE – Concluding remarks and future perspectives

Secretion systems of Soft Rot *Pectobacteriaceae* are involved in bacteria-bacteria, bacteria-environment, and bacteria-host interactions that lead to disease symptom development and progression (Charkowski *et al.*, 2012). Knowledge of secretion systems in *Pectobacterium brasiliense* 1692 (*Pbr*1692) is largely obscure, a limitation that needs to be overcome to allow for the development of novel antimicrobial strategies to target them. Against this background, the primary role of this study was to elucidate the roles of two understudied secretion systems, namely outer membrane vesicles (OMVs) and the type 6 secretion system (T6SS), through proteome analysis and characterisation. This report lists the proteins associated with OMVs and the T6SS of *Pbr*1692 in rich and virulence gene-inducing media, respectively. *Pbr*1692 produces two forms of membrane vesicles that harbour and associate with diverse and different cargo that is linked or predicted to have varied functions including biofilm formation, nutrient uptake, antibiotic resistance, competition, host tissue damage, and elicitation of the hypersensitive response (HR) (Chapter Two, Table S2.1, Figures 2.1D, 2.5-2.8). Of note, some OMV cargo were predicted to be substrates of other canonical secretion systems of Gram-negative bacteria (GNB), highlighting an overlap between the vesicles and secretion systems of GNB. On the other hand, the study identified proteins associated with T6SS-active and -inactive strains of *Pbr*1692 (Chapter Three, Table 3.2 and Table S3.1). These included differentially expressed and secreted proteins (DSPs) by the *Pbr*1692 T6SS-inactive strains compared to the T6SS-active strains (Figure 3.2, 3.3, 3.4). Compared to the predicted cargo of the secretion systems of *Pbr*1692, there was an indication that the T6SS, similar to OMVs shares functionally diverse cargo with other secretion systems (Table 3.3). *Pbr*1692 T6SS potentially shares most substrates with T2SS, the primary virulence determinant of Soft Rot *Pectobacteriaceae* (SRP). In addition to the secretion system studies, we have shed light on a conserved SRP virulence factor, Avirulence protein L, and its potential role in the ability of *Pbr*1692 to withstand host defences (Chapter 4). This protein serves as an example of a shared substrate between the OMVs, T2SS, and putatively with T6SS, thus a good candidate target for *Pbr*1692 control. Never has AvrL been associated with other systems besides the T2SS.

Recently, Joshi and co-workers reported that metabolites of a wild potato provide defence against *P. brasiliense* by reducing the enzymatic activities of T2SS pectinase, cellulase, and protease enzymes (Joshi *et al.*, 2021, Joshi *et al.*, 2022). Management strategies that target secretion systems and their substrates may prove useful indeed. Proteins facilitate the dialogues and the responses on both ends of the interaction between the pathogen and the host, as discussed in Chapter One. It is unclear to what extent system-level interactions influence the response of pathogens like *Pbr1692* to their respective environments. We report here that OMVs protect some T2SS secreted enzymes in their lumen (Chapter Two, Figure 2.7). OMVs have been an unstudied resource in *Pectobacterium* spp. aggression that cannot be overlooked (Jonca *et al.*, 2021). In most cases, such secondary secretion is negated by the focus on the primary secretion systems. Thus, we propose targeting inter-systems secretion in developing management schemes of *Pbr1692*.

Future perspectives for this work should seek to validate further the potency and influence of *Pbr1692* OMVs in microbe-microbe and *in planta* host-pathogen interactions. This will expand our knowledge regarding OMVs as a formidable inter-secretion instrument in the survival and virulence strategy of *Pbr1692*. As such, agents that suppress OMV production should be investigated. To further study substrate co-secretion by OMVs, T1SS-T4SS, and T6SS, reporter-based assays can be used. Guided by the secretion systems under inspection, substrates would be cloned as fusions with detectable tags that do not impact secretion (i.e., neutral tags) and expressed by transformed strains for selective identification using affinity chromatography, fluorescence microscopy, enzymatic assays, or Western blot from the wild type and single or double secretion system inactive strain secretomes. Tags associated with an enzymatic activity provide quantitative data on secretion. Densitometry can be used for the quantification of relative band intensities.

The internalisation of *Pbr1692* OMVs by the plant cell wall and the OMV induced resistance of host immunity is an area that this study has set path towards. The lack of OMV mutants, however, could prove challenging as there are no OMV mutants for comparison to establish OMV induced immunity against mutant controls (Katsir & Bahar, 2017). Despite this hurdle, we anticipate isolated OMVs can still be used to determine pathogen benefit in pathogen plus isolated vesicles (i.e., vesicle

supplementation) and pathogen-only scenarios. Also, as in *Xanthomonas fastidiosa* (Ionescu *et al.*, 2014), conditions that lead to hyper vesiculation can be investigated and used to observe plant responses or OMV benefit to the pathogen *in planta*. OMVs in biofilm-like matrices identified in this study were formed by a planktonic culture. It is probable that *Pbr1692* OMVs are more inclined to participate in biofilm formation. It would be especially interesting to investigate the physiological and ecological roles of *Pbr1692* biofilm OMVs and cargo.

Apart from follow-up investigations such as quantitative tests to establish OMV potency e.g., in competition, there are experimental reports that show evidence that vesicles mediate the transport of RNA and DNA with major advantages compared with conventional secretion (Guerrero-Mandujano *et al.*, 2017). In addition, chromosomal, plasmid, or phage DNA, and varied forms of RNA have been identified in OMVs (Orench-Rivera & Kuehn, 2016, Bitto *et al.*, 2017, Tsatsaronis *et al.*, 2018, Lee, 2019). Packaged material is ferried over long distances and protected from degradation, which also implies that these contents are not diluted (Kulp & Kuehn, 2010). *Pbr1692* produces vesicles with double membranes (O-IMVs and EOMVs) that provide extra protection to protease and nuclease sensitive cargo (Chapter Two, Figure 2.3). Although few, the double membrane vesicles warrant investigation to trace the genetic material in OMVs and establish the extent to which OMVs facilitate dissemination of genes such as antibiotic resistance genes and other genetic information between cells via horizontal transfer of nucleic acids (Rumbo *et al.*, 2011, Domingues & Nielsen, 2017, Lynch & Alegado, 2017). More so, OMV small RNAs could serve as a tool for regulating other microbes and plant responses to infection by obstructing mRNA translation (Tsatsaronis *et al.*, 2018). Appropriate stimuli (e.g., temperature or antibiotic stress) can be determined to increase vesiculation, nucleic acid concentrations and subsequent packaging, and transfer by OMVs (Fulsundar *et al.*, 2014). The study of genetic material shared between microbial communities is particularly important given that recent evidence in *Cupriavidus necator*, suggests OMV recruitment via the T6SS for HGT during interspecies communication (Li *et al.*, 2021). Furthermore, T6SS-mediated death of rival cells residing in a biofilm could be speculated to supply the much-needed genetic material by releasing DNA that can later be exchanged between killer cells via OMV-based HGT. Therefore, the potential

for T6SS/OMV intersecretion activity in the context of bacterial HGT via the recruitment of OMVs should also be investigated in *Pbr1692*.

Bacterial vesicles can also serve as potential ‘biocontrols’, meaning they have the potential to be established as an ideal delivery platform for a range of biocontrol agents. Uptake of *Pbr1692* OMVs by phytopathogen *Phytophthora parasitica* zoospores was observed recently in an unpublished study in our laboratory. The development of synthetic vesicles with regard to biocontrol will also be highly influenced by research into the biology of bacterial vesicles and their efficacy. It has been proposed that the first step in appreciating vesicle mediated benefits to plant-microbe interactions is primarily through OMV isolation and characterisation, which we have done. In another instance, purified OMVs from bacteria have been shown to act as a ‘vaccine’ whereby they trigger plant defences against bacterial and oomycete pathogens (McMillan *et al.*, 2021). Indeed, the exogenous use of OMVs as immune response triggers against plant infectious diseases or as priming agents for enhanced field performance presents an intriguing opportunity (Motaung & Steenkamp, 2021). A recent study showed that bacteria can package into OMVs cytoplasmic GFP protein and β -lactamase originating from a plasmid not native to the OMV producing cells (Jonca *et al.*, 2021). Therefore, it would be beneficial to consider vesicles of highly competitive bacteria such as *Pbr1692* for enhanced toxin delivery against those of existing biocontrols. This has a greater potential given the vesicles would contain diversified concentrates of active antimicrobials that would last longer in the environment (Bomberger *et al.*, 2009). The advantage of OMVs is in their constitutive expression and ability to transport many molecules simultaneously to the target sites (Li *et al.*, 2016, Reidl, 2016, Katsir & Bahar, 2017, Ahmadi Badi *et al.*, 2020). Adaptation of OMVs into chemical-free alternatives to shape microbial communities and prime plant immunity for crop benefit is a promising avenue for sustainable agriculture (Borrero de Acuña & Bernal, 2021).

In this study, we predicted that among OMV/T6SS shared cargo were putative virulence factors. The T6SS has in most cases been associated with competition, including in *Pbr1692* (Shyntum *et al.*, 2019). In fact, since 2008, there has been no other study on a *Pectobacterium* sp. T6SS in association with virulence. Notwithstanding, T6SS virulence studies have been conducted in other plant

pathogens (Tian *et al.*, 2017, Asolkar & Ramesh, 2020, Kim *et al.*, 2020). In light of these studies, the research community has become aware that the T6SSs function in a species-specific manner. For example, *Xanthomonas oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* both infect rice but the T6SS of *X. oryzae* pv. *oryzae* is involved in virulence while the T6SS of *X. oryzae* pv. *oryzicola* is not (Choi *et al.*, 2020, Zhu *et al.*, 2020). This has also shown that T6SS association with the virulence of phytopathogens should remain an active research topic. With the turn that our research had taken (i.e., with OMV/T6SS candidates associated with virulence), the total proteome of *Pbr1692* was screened to identify 49 T6SS associated proteins under virulence gene-inducing conditions *in vitro* (Chapter Three). Four candidate virulence factors predicted to be shared between OMVs and T6SS were identified in the T6SS-active secretome as DSPs. In the call to expand our understanding of the T6SS to uncover new effectors involved in modulating plant-microbe interactions, we should, in future, functionally characterise all 19 active T6SS candidates (Table 3.2) that presently have poor annotation scores in UniProt. The first point of study would be phenotypes of *Pbr1692* mutants of the major exported protein, VgrG and HNH endonuclease that were the top three T6SS substrates based on spectral counts. The next target group will compose of the other ten predicted and *in vitro* validated T6SS substrates superoxide dismutase, Hcp, VgrG, extracellular solute-binding protein, alpha/beta fold hydrolase, alginate lyase, peptidyl-prolyl cis-trans isomerase, and formate C-acetyltransferase. Apart from T6SS substrates, it is worth investigating why certain proteins are only secreted by T6SS-inactive strains and what roles they play.

In Chapter Four, Avirulence protein L was chosen for further study based on its abundance in OMVs (Maphosa & Moleleki, 2021), previous research showing it is a *Pectobacterium* sp. T2SS substrate (Corbett *et al.*, 2005), and its prediction as a shared T2SS/T6SS substrate via BastionX (Chapter Three, Table 3.1). However, it was not differentially secreted in the T6SS-inactive strain compared to the T6SS-active strain, suggesting that its secretion via T6SS might have been a false prediction. We dismissed its potential as a T6SS substrate as we could not validate this claim *in vitro*. Therefore, despite this work being of great value, it was faced with a significant challenge to highlight the crosstalk between the three secretion systems OMVs, T2SS, and T6SS using AvrL. Along with this arose some questions that are of interest to enriching the findings of this research and perhaps to the broader community:

- Is there any compensatory behaviour between OMVs, T2SS, and T6SS?
- What type of secretome profiles would we encounter when more than one secretion system is knocked out?

Despite AvrL's absence in the T6SS associated proteome, we expanded the current knowledge on this protein, including the finding that there is a high probability that it is shared between T2SS and T6SS, indicated by its prediction score improving from ~0.9 to 1 BastionX. Due to the fact that OMVs and the T2SS were active in the T6SS-inactive strain, T2SS and OMV may have masked the lack of the T6SS contribution in the mutant. Most importantly, under non-stressful conditions studied in Chapter Two, the abundance of proteins such as AvrL in the vesicles suggest they are an important resource. Our *in silico* analysis predicted that AvrL is a zinc binding metalloprotease partially functionally linked to some T2SS substrates (Figures 4.4 and 4.6). In studies conducted on *Vibrio* and *Pseudomonas* spp., neutral zinc metallopeptidases with the same HEXxH zinc binding motif as AvrL were identified only in T6SS-inactive secretomes (Hood *et al.*, 2010, Altindis *et al.*, 2013). Interestingly, T6SS component proline alanine alanine arginine (PAAR) proteins with a role in recruiting effectors also have conserved zinc binding sites with histidine residues (Shneider *et al.*, 2013).

Sequence analysis also revealed that the *Pbr1692* AvrL protein contains seven intrinsically disordered regions (IDRs) and part of a conserved putative lpxD enzyme domain in the longest IDR. LpxD enzymes are involved in the biosynthesis of lipid A, a constitutive component of lipopolysaccharide (LPS) crucial for optimal growth, survival, and virulence of most GNB including *Pectobacterium* spp. (Evans *et al.*, 2010). The LPS is also a major structural component of OMVs. OMV-mediated transport and trade of the LPS to host cells and among bacterial species has been reported (Vassallo *et al.*, 2015, Vanaja *et al.*, 2016, Glew *et al.*, 2021). *Pectobacterium* spp. LPS induces cell death of *Arabidopsis thaliana* cells, a quality not attributed to non-pathogenic *Escherichia coli* or *Pseudomonas aeruginosa* LPS (Mohamed *et al.*, 2015). Although this characteristic is not yet entirely attributed to the lipid A moiety in plant cell death (PCD), it is worth considering lpxD enzymes as drug targets of multitarget inhibitory peptides. This way, the peptides would disrupt the early stages of lipid A biosynthesis (and consequently LPS biosynthesis or anchoring) in treatment combinations for soft rot disease (Jenkins *et al.*, 2012, Jenkins *et al.*, 2014). The same

goes for the recently validated metallopeptidase HExxH motif in Sv_x (Duarte *et al.*, 2016, Hasan *et al.*, 2021, Tendiuk *et al.*, 2022). We already anticipate that AvrL, similar to Sv_x mutants will be compromised in virulence; thus, targeting IpxD and metallopeptidase enzymes could be a step closer to controlling *Pbr1692*. Validation of the IpxD domain activity is particularly important given the conserved insertions and repeat observed in SRP IpxD.

Interactomics and functional predictions showed that AvrL is influential in several host cellular processes including defence. In this study, we predicted that 19 proteins are potential targets of AvrL in susceptible *S. tuberosum* cultivar Valor and hypothesise that AvrL-intracellular host protein interactions occur (Figure 4.8 and S5.1). More so, this links the *in planta* upregulation of *Pbr1692* AvrL and the differentially expressed defence genes of *S. tuberosum* cv. Valor challenged with *Pbr1692*. Nothing is as simple as it looks when it comes to bacterial evolutionary processes and the outcomes of the qualities they pick up in their niches. We have much to learn and this work shows so much potential for extendibility in the quest to understand *Pbr1692* pathogenicity.

Our findings provide precedence for further inquiry. Interaction is the most fundamental function of proteins and underpins all other functions and spheres of influence of a protein. Therefore, more work needs to be done for the findings to be conclusive. We have the task of validating the predicted interactions and determine the degree of specificity and regions facilitating interactions between AvrL and the proposed host interactors identified in the study. Presently, there is no solved structure of AvrL or adequate template in homology-based modelling tools. To computationally mimic the orientations of interaction, the AvrL and its targets should be modelled *de novo* and docked. The docking scores from the simulations can then be used to screen for targets with the most stable interactions. More so, this will be instrumental in determining if the domains or IDRs of AvrL participate in the interactions. *In vitro*, pairwise yeast 2 hybrid assays (Y2H) can be used to validate interaction between AvrL and the candidates. AvrL would be fused to the N-terminus of the DNA binding domain of a yeast GAL4 transcription activator and cloned to bait the AvrL candidates fused to the C-terminus of the activation domain of GAL4 in co-transformed yeast clones. Interaction can be verified by the reconstitution of the transcription factor and activation of the reporter gene. The co-localisation of AvrL with its validated interactors should

also be confirmed. We have cloned AvrL using Gateway cloning technology for *Agrobacterium* mediated transient expression of the GFP tagged AvrL in *Nicotiana benthamiana*. The Y2H validated targets can be tagged using a different fluorescent protein and co-expressed with AvrL and visualised using confocal microscopy.

Overall, our group's work on *Pbr1692*, with the overall goal to understand the virulence determinants it uses to adapt and cause disease in potatoes and the regulatory networks that govern its virulence factors, dates back more than eight years. This was our first study on *Pbr1692* to be conducted at the proteome level, focusing on two secretion systems and the potential crosstalk between secretion systems of *Pbr1692* in general. Studies have shown that OMVs transport cargo of other secretion systems (Guerrero-Mandujano *et al.*, 2017). This is broadly stressed in Chapter One, where we also propose that there could be an overlooked extent of potential substrate overlap between secretion systems that should be considered in bacterial interactions. For this reason, we have surveyed the literature at our disposal and carried out this study to pioneer understanding of inherent species-specific crosstalk and coordinated response between secretion systems. Future studies should work towards demonstrating that these secretion system intersections impact a range of processes that are ecologically important to bacteria, including virulence, nutrient acquisition, attack, defence, horizontal gene transfer (HGT), and host manipulation. We hope that we have, therefore, steered interest towards the implications of the inter secretion system-mediated responses in bacterial activities, pathogen control, and disease management strategies.

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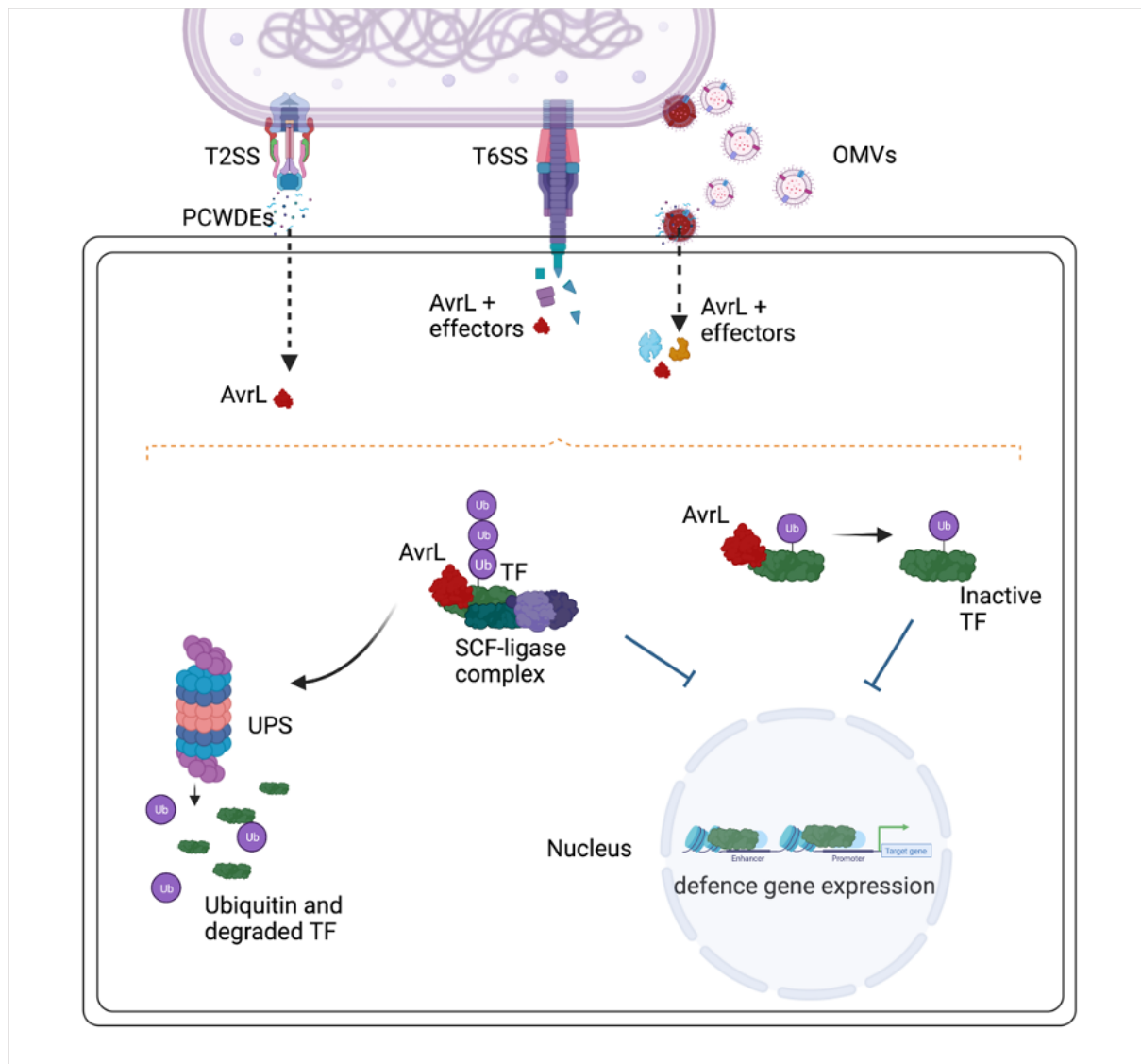


Figure 5.1: A hypothetical model showing the type 2 secretion system (T2SS), the type 6 secretion system (T6SS), and outer membrane vesicles (OMVs), all possibly secreting Avirulence L. The T6SS directly introduces effectors into the cell, AvrL and co-existing plant cell wall degrading enzymes break down the cell wall, and OMVs attach to the plant membrane to transfer AvrL, enabling AvrL entry into the plant cell. Once inside the cell, AvrL interacts with a number of host proteins, including transcription factors (TF) expressed in response to the *Pectobacterium brasiliense* 1692 infection. These interactions might either target the host proteins for degradation via the ubiquitin proteasome system (UPS) or inactivate them.