Genetic profiling of the interactions between soft rot *Pectobacterium* species and plants

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ACADEMIC DISSERTATION

To be presented for public examination with the permission of the Faculty of Biological and Environmental Sciences of the University of Helsinki in the lecture room LS7, Latokartanonkaari 7 (B-building), Helsinki, on the 20th of March 2015, at 12 o'clock noon.

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ISSN 2342-5423 (print)
ISSN 2342-5431 (PDF)
ISBN 978-951-51-0835-7 (print)
ISBN 978-951-51-0836-4 (PDF)
http://ethesis.helsinki.fi
Hansaprint 2015

“There are two kinds of scientific progress: the methodical experimentation and categorization which gradually extend the boundaries of knowledge, and the revolutionary leap of genius which redefines and transcends those boundaries. Acknowledging our debt to the former, we yearn nonetheless for the latter.”

Academician Prokhor Zakharov, Sid Meier’s Alpha Centauri

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I Nykyri J*, Niemi O*, Koskinen P, Nokso-Koivisto J, Pasanen M, **Broberg, M**, Plyusnin I, Törönen P, Holm L, Pirhonen M, Palva ET. 2012. Revised Phylogeny and Novel Horizontally Acquired Virulence Determinants of the Model Soft Rot Phytopathogen *Pectobacterium wasabiae* SCC3193. PLoS Pathog. 2012;8(11):e1003013. doi: 10.1371/journal.ppat.1003013. Epub 2012 Nov 1.

II Kõiv V, Andresen L, **Broberg M**, Frolova J, Somervuo P, Auvinen P, Palva ET, Pirhonen M, Tenson T, and Mäe A. 2013. Lack of RsmA induces constant hypervirulence, cell elongation and hyperflagellation in *Pectobacterium wasabiae*. PLoS One. 2013;8(1):e54248. doi: 10.1371/journal.pone.0054248. Epub 2013 Jan 23.

III **Broberg M**, Lee GW, Nykyri J, YH Lee, Pirhonen M, Palva ET. 2014. The global response regulator ExpA controls virulence gene expression through RsmA-mediated and RsmA-independent pathways in *Pectobacterium wasabiae* SCC3193. Appl Environ Microbiol. Mar 2014; 80(6): 1972-1984, doi: 10.1128/AEM.03829-13.

IV **Broberg M***, Davidsson P*, Kariola T, Witick V, Sipari N, Heino P, Pirhonen M, Palva ET. Short oligogalacturonides induce pathogen resistance-associated gene expression in *Arabidopsis thaliana*. 2015. *Manuscript*.

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I) MB designed and constructed the T6SS double mutant and wrote the materials and methods for that experiment. MB participated in designing and performing the potato tuber virulence assays and growth curve measurements together with JN and ON.

II) MB participated in writing the microarray part of the Materials and Methods section and designed and performed the microarray hybridization together with LA. MB performed the analysis of the microarray data with PS.

III) MB designed and conducted all of the experiments. MB participated in analyzing the microarray data and analyzed all other data. MB wrote the manuscript with ETP and MP.

IV) MB participated in the design and analysis of all experiments together with PD. In addition, MB performed the RNA sequencing expression analysis, quantitative RT-PCR, virulence assays, bioinformatics and growth retardation assays together with PD and TK. MB and PD analyzed all of the experimental data. MB wrote the manuscript together with PD, TK, MP and ETP.

ABBREVIATIONS

ABA	Abscisic acid
ABC	ATP-binding cassette
AHL	N-acyl homoserine-lactone
aRNA	antisense RNA
Avr	Avirulence
Cel	Cellulase
DAMP	Damage-Associated Molecular Pattern
DM	Double Mutant
DP	Degree of Polymerization
EPS	Exopolysaccharide
ET	Ethylene
ETI	Effector Triggered Immunity
exp-rsm	Regulatory pathway with *expSA* and *rsmBA*
GO	Gene Ontology
GSEA	GeneSet Enrichment Analysis
glu-SA	Glucosyl Salicylic Acid
HGT	Horizontal Gene Transfer
HR	Hypersensitive Response
HSL	Homoserine Lactone
JA	Jasmonic Acid
MAPK	Mitogen-Activated Protein Kinase
MS	Murashige Skoog
ncRNA	non-coding RNA
NO	Nitric Oxide
OGs	Oligogalacturonides
PAMP	Pathogen-Associated Molecular Pattern
PCD	Programmed Cell Death
PCWDE	Plant Cell Wall Degrading Enzyme
Peh	Polygalacturonase
Pel	Pectate lyase
Pem	Pectin methyltransferase
PGA	Polygalacturonic acid
Pnl	Pectin lyase
Prt	Protease
PTI	PAMP-Triggered Immunity
RT-qPCR	Reverse Transcription quantitative Polymerase Chain Reaction
QS	Quorum Sensing
R	Resistance
RLK	Receptor-Like Kinases
ROS	Reactive Oxygen Species
RT	Reverse Transcription
SA	Salicylic Acid
SAGE	Serial Analysis of Gene Expression
SAR	Systemic Acquired Resistance

T2SS	Type 2 Secretion System
T3SS	Type 3 Secretion System
T4SS	Type 4 Secretion System
T6SS	Type 6 Secretion System
TCS	Two-Component System
TF	Transcription Factor
Xyl	Xylanase

ABSTRACT

The interactions between phytopathogenic bacteria and their host plants can be characterized as an intricate web of signals and appropriate responses. Phytopathogenic soft rot bacteria occur globally, causing disease in *Solanum tuberosum* (potato) and other tubular staple foods in both the field and storage. One widely studied soft rot bacterium is *Pectobacterium wasabiae*, which has been identified in *Eutrema wasabi* (wasabi) plants in Japan and in potatoes in Finland. Generally, the interactions between this type of bacterium and host plants are characterized by maceration of plant tissue, due to the actions of secreted plant cell wall degrading enzymes (PCWDE), and the induction of phytohormone dependent defenses in the plants. The maceration of plant tissue involves the release of pectic oligogalacturonides (OGs) from plant cell walls. OGs have been identified as important signaling compounds, inducing the expression of a variety of defense-related genes. As the bacterial infection advances, the bacteria coordinate the production of virulence factors by utilizing regulatory proteins that modulate the transcriptome. Transcriptomic analyses have been used extensively in past studies to identify regulatory networks and signaling pathways, and these studies have provided insights into the processes underlying plant-pathogen interactions.

The novel scientific results of this dissertation are derived from a combination of transcriptomic, genomic, genetic, and phenotypic analyses. This study analyzed various aspects of plant-pathogen interactions. The central bacterial model used was *P. wasabiae,* and the model plant of interest was *Arabidopsis thaliana.*

This study characterized the genome of *P. wasabiae* via sequencing and bioinformatics analysis. Various virulence associated genes and operons, such as two distinct type 6 secretion systems, were identified and annotated. The bacterium was found to in fact be more related to *P. wasabiae* than *Pectobacterium carotovorum*, which the strain originally had been named after. Furthermore, a combination of functional genetics and transcriptomic methods, such as reverse transcription quantitative PCR (RT-qPCR) and microarrays, were used to determine the regulons controlled by the proteins ExpA and RsmA in *P. wasabiae*. These two proteins have been identified as important for the virulence of several γ-proteobacterial pathogens. This study analyzed the regulons via the use of three mutants: *expA, rsmA*, and an *expA rsmA* double mutant (DM). Overlapping and independently regulated targets were identified between ExpA and RsmA. Phenotypic assays for motility, growth, PCWDE activity, and virulence confirmed the transcriptomic data for the mutant strains. Novel findings included reduction of swimming motility in agar medium for *P. wasabiae expA* and *rsmA* mutants. In addition, the DM exhibited enhanced virulence and fitness *in planta* compared to either single mutant. Via analysis of transcriptomic data, a subset of genes was identified as affected in expression by an *expA* mutation independently of the presence of *rsmA*.

The relatively unexplored role of short OGs (with a degree of polymerization (DP) <10) in damage-associated molecular pattern (DAMP) signaling in *A. thaliana*

was characterized in this study. Comparative gene expression profiling based on RNA sequencing and RT-qPCR was performed on RNA harvested from plants treated with short OGs or with a mock suspension. Phenotypic assays confirmed the gene expression data. In a meta-data analysis, the resulting RNA sequencing and RT-qPCR data were compared with gene expression data from previous studies, in which long OGs (DP>10) were used to treat plants. This work demonstrated that short and long OGs induce genes and genesets associated with pathogen defense and phytohormone signaling, whereas reducing plant growth and development. The transcriptomic data of this study suggests that plant treatment with a mixture of short or long OGs yields a more pronounced and varied modulation of global gene expression, compared to treatment with only trimeric OGs. The regulation of the virulence of *P. wasabiae*, and the DAMP signaling triggered by plant cell wall damage in *A. thaliana*, are elements of the interactions between the plant and pathogen. The studies presented in this dissertation provide novel information about these two biological processes and highlights their connection.

1 INTRODUCTION

1.1 Transcriptomics as a tool for understanding plant-microbe interactions

1.1.1 Relevance of the transcriptome

Cells constantly process information from their external and internal environments. Signals are received and responses are integrated at various levels of the intracellular information flow. The so-called “central dogma” of molecular biology depicts the transfer of information from DNA to RNA to proteins within biological systems, combined with the replication of DNA (Piras et al., 2012). To organize the information from environmental signals and to adapt to their environment, cells utilize complex regulatory networks. Of particular interest for this study is the mid-point of information transmission from genes to gene products: the RNA population of cells. Transcriptional analysis can provide important insight into the activity of biological pathways and gene regulation. Thus, transcriptomics functions as an important tool for elucidating the mechanisms by which cells with identical genomes behave differently, depending on differing intra- and inter-cellular environments.

During the infection of plants by bacteria, signal cascades are processed, causing a fluctuation in gene expression and the modulation of transcriptional regulation. The existing RNA molecules in a cell, or in a population of cells, represent a response to the signals that the cell has received, and their encoded messages affect the forthcoming reactions. Regulation at this stage of the information flow serves as an important focal point for controlling and tuning cellular reactions. Thus, observing the total RNA in a cell, or a

population of cells, at a certain point in time provides a map of the past, present and future of that biological system.

1.1.2 Transcriptional analysis methods

The earliest type of transcriptomic analysis was based on the extraction of total RNA from cells, combined with the candidate gene-based Northern blotting technique (Hirai et al., 2005; Morozova et al., 2009; Kautza and Zuckerbraun, 2014). Northern blotting is a widely used low-throughput method that is time-consuming, labor-intensive and requires relatively large amounts of input RNA. Radiolabeled probes are made for the transcripts of interest, and they are bound to complementary messenger (or other) RNA species, which are initially separated by their size via gel electrophoresis and transferred onto a solid surface. This method allows the measurement of transcript size and enables relative comparisons of expression. Following Northern blotting, RT-qPCR-based methods have been adopted and are now commonly used for relative gene expression analysis (Kautza and Zuckerbraun, 2014; Morozova et al., 2009). Complementary primers are used for the amplification of target transcripts in cDNA. The amplification can be measured in real-time using fluorophores that bind to the amplified DNA. The RT-qPCR method has significantly increased the amount of output data from RNA samples. However, RT-qPCR is generally not viewed as suitable for the large-scale analysis of whole transcriptomes, which are usually in the range of thousands of gene products.

For the purpose of global transcriptomic analyses, microarrays are a standard method for assessing gene expression and provide a means to analyze thousands of predicted transcripts simultaneously (Feichtinger et al., 2012; McGettigan, 2013; Morozova et al., 2009; Wang et al., 2009). The technique relies on the synthesis of cDNA, or in more modern applications antisense RNA (aRNA), from a harvested RNA population. The cDNA or aRNA is subsequently hybridized to oligonucleotide probes corresponding to transcripts of interest. The amplification of a relatively small amount of purified mRNA to aRNA is now a commonly used method in comparison to the more classical techniques based on the total RNA of samples (Kelz et al., 2002). The cDNA or aRNA is attached to a fluorescent dye, and the subsequent comparative transcriptome analysis is based on emitted fluorescence that depends on the amount of probe-bound transcripts. The problems of isoform transcripts (mRNAs originating from the same locus but with different coding sequence) and the detection of small RNAs (sRNA) and non-coding RNAs (ncRNA) have been partially solved via tiling arrays that cover the entire genome of an organism. Thus, the analysis of tiling array data requires fewer assumptions regarding the nature of the transcriptome (Malone and Oliver, 2011; Morozova et al., 2009; Mortazavi et al., 2008). However, limitations remain for the technique, including background signal noise, assumptions regarding the organism's genome (which may not be completely identified), and cross-hybridization of transcript fragments with complementary sequence to probes of different genes (Wang et al., 2009). The rise of next-generation RNA sequencing addresses many of the shortcomings of hybridization-based methods, as well as older sequencing-based methods, such as serial

analysis of gene expression, commonly abbreviated as SAGE (Morozova et al., 2009). The present sequencing methods are mainly based on PCR amplification of fragment libraries, combined with the measurement of fluorophores anchored to the termini of oligonucleotides used for amplification (Malone and Oliver, 2011; Morozova et al., 2009). Using the latest generations of sequencers, transcriptomic information can be gained without the need for a reference genome (Morozova et al., 2009). Highly accurate transcript measurements can be obtained via sequencing, especially via the use of samples spiked with a known amount of a particular transcript. Furthermore, different isoforms and RNA splice junctions can be distinguished efficiently, particularly when compared with microarrays and SAGE (Mortazavi et al., 2008).

High-throughput sequencing is currently undergoing rapid development. The cost of sequencing global transcriptomes has remained relatively high when compared with microarrays, and the wet lab routines contain time-consuming library construction. The construction of the library may also introduce bias and artifacts in the sequencing (Quail et al., 2012; Shendure and Ji, 2008; van Dijk et al., 2014; Wickramasinghe et al., 2014). Furthermore, sequencing is not completely free of its own data ambiguities that result from similar transcript sequences or DNA polymerase synthesis errors (McGettigan, 2013; Wang et al., 2009). The vast amounts of data generated by sequencers also pose a challenge for the future, both in terms of storage and resources for analyses (van Dijk et al., 2014; Wickramasinghe et al., 2014; Zhao et al., 2014). Microarrays, with their well-developed analysis pipelines and low cost, are still regarded as viable candidates that are comparable to current sequencing methods. Microarrays and RNA sequencing remain fairly comparative in relative gene expression analyses when studying organisms with well-characterized transcriptomes (Feichtinger et al., 2012; Malone and Oliver, 2011; Zhao et al., 2014). However, as technology and analysis algorithms are improved to compensate for sequencing limitations, computing power, and equipment costs, the current trends point toward the inevitable domination of sequencing technology (Black et al., 2014; Zhao et al., 2014).

1.2 Soft rot bacteria

1.2.1 Plant pathogenic γ-proteobacteria

Phytopathogens are normally classified as biotrophs or necrotrophs. Biotrophic organisms are characterized by a lifestyle of leeching nutrients from host cells without killing them, whereas necrotrophs kill host cells in order to survive and propagate (Collmer et al., 2009; Lotze et al., 2007). Many γ-proteobacterial phytopathogens, such as *Pseudomonas syringae* and *Xanthomonas campestris*, are hemibiotrophic pathogens that initially require living tissue. This initial biotrophic phase is then followed by a shift to a more necrotrophic mode that is accompanied by the degradation of host cells as the invasion progresses (Lee and Rose, 2010; Liu et al., 2008; Üstün et al., 2013). To achieve a biotrophic lifestyle, the bacteria are required to stealthily avoid detection and neutralization by the host's defense mechanisms. The stealth-based infection is commonly achieved by the use of plant metabolite analogs and

proteins termed effectors (Kay and Bonas, 2009; Lindeberg et al., 2012; Liu et al., 2008; Toth and Birch, 2005; Üstün et al., 2013). Effectors are proteins that inhibit different plant pathogen resistance pathways and cellular functions in order to promote bacterial proliferation (Lindeberg et al., 2012). In contrast, the necrotrophic lifestyle featured by γ-proteobacterial plant pathogens, such as the *Pectobacterium* species, is associated with forceful maceration of host tissue via the release of digestive enzymes and toxins, potentially requiring a less active manipulation of the host's defense responses (Liu et al., 2008; Toth et al., 2006; Toth and Birch, 2005). The digestive enzymes utilized by plant pathogenic bacteria during their necrotrophic phase to degrade macromolecular complexes of the host are commonly referred to as plant cell wall-degrading enzymes, abbreviated as PCWDEs (Kariola et al., 2003; Marits et al., 2002; Toth et al., 2006).

1.2.2 Common plant cell wall-degrading enzymes of necrotrophic pathogens

As necrotrophic bacteria commence the infection of the plant host, they utilize PCWDEs to macerate the plant tissue to obtain nutrients for further propagation. The PCWDEs include a variety of proteins that catalyze the breakdown of pectin, pectate, proteins, and cellulose. This breakdown of cell wall components by PCWDEs leads to plant cell lysis and tissue maceration (Lee et al., 2013). Pectin, pectate, cellulose, and proteins together form important structures of the plant cell wall and the lamella between the cells; these structures are all important sources for the bacteria to extract their nutrition during infection (Toth et al., 2006; Toth and Birch, 2005). Proteases (Prt) degrade proteins. Pectinolytic enzymes, or pectinases, degrade pectin and pectin derivatives and include enzymes, such as pectin lyases (Pnl), pectate lyases (Pel) and polygalacturonase (Peh). The different categories of pectinolytic enzymes operate at different levels during the degradation of pectin (Abbott and Boraston, 2008; Chatterjee et al., 1995; Liu et al., 1999; Pirhonen et al., 1991). Pectin is one of the three main polysaccharides that form the plant cell wall, and it acts to connect cellulose and hemicellulose fibers (Abbott and Boraston, 2008; Micheli, 2001). The Peh enzymes digest by hydrolytically cleaving α-1,4-galacturanosyl links that make up the pectin and pectate polymers, whereas Pels and Pnls operate by β-elimination (Alfano and Collmer, 1996; Barras et al., 1994). In addition to the enzymes directly attacking the links between the galacturonan residues that make up pectin, pectin methylesterases (Pem) attack the methyl side chains of pectin, causing destabilization of the cell wall integrity (Barras et al., 1994; Micheli, 2001). The other main components of the plant cell wall are cellulose and hemi-cellulose, which can be digested by cellulases (Cel) and expansins. Cellulases, also known as endoglucanases, hydrolyze the 1,4-β-D-glycosidic links of cellulose and hemi-cellulose, producing derivatives, such as cellobiose (Barras et al., 1994). However, expansins cleave non-covalent bonds between cellulose microfibers and other polysaccharides, opening up the matrix for further degradation via a non-hydrolytic mechanism (Kim et al., 2009; McQueen-Mason and Cosgrove, 1995). Other PCWDEs include phospholipases and xylanases (Xyl) (Barras et al., 1994). Thus, the PCWDEs are a diverse collection of proteins, each with a specific target of the plant cell wall (Figure 1).

The type 1 secretion systems (T1SS) and type 2 secretion systems (T2SS) are common among γ-proteobacteria and are essential for the secretion of the PCWDE arsenal into the extracellular space (Glasner et al., 2008; Toth et al., 2006). Prt are generally secreted by the T1SS. The export of the majority of the PCWDEs is performed via the T2SS encoded by the *out* operon (Alfano and Collmer, 1996; Barras et al., 1994; Reeves et al., 1993; Salmond, 1994; Sandkvist, 2001). The export of these proteins from the cytoplasm to the periplasm starts with the Sec system, and then the proteins are transported over the outer membrane by the T2SS (Rodríguez-Sanz et al., 2010). The function of T2SS as a delivery system is an integral part of the controlled transport of functional PCWDEs to the extracellular space for many necrotrophic plant-pathogenic bacteria; thus, T2SS is essential for virulence.

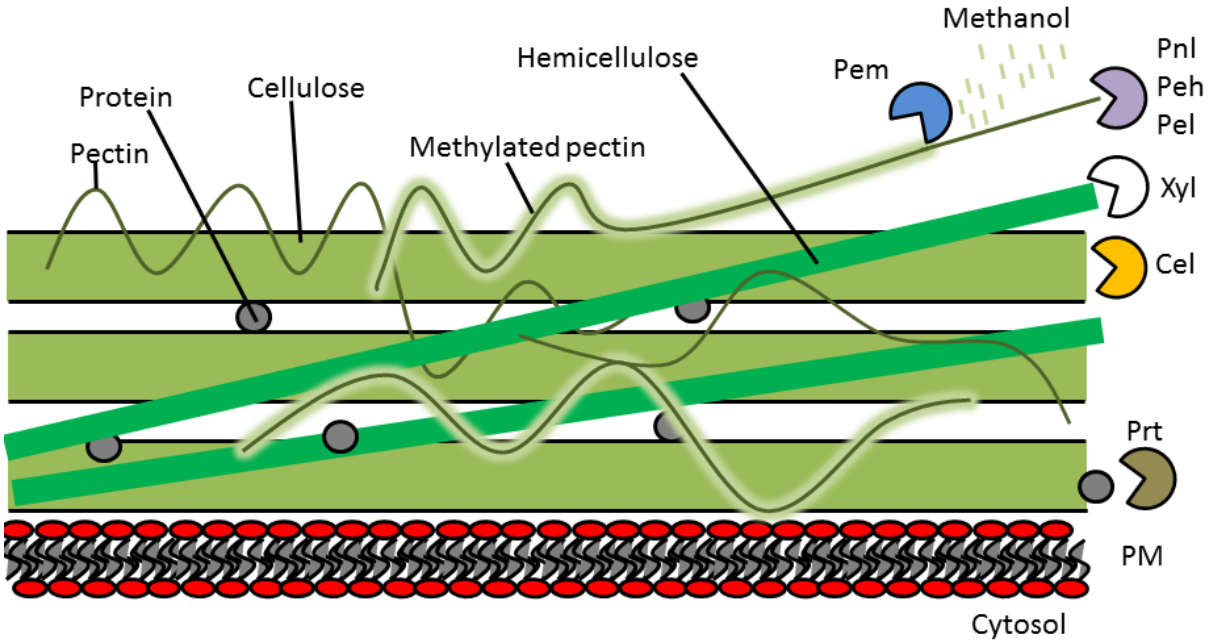


Figure 1. The primary types of plant cell wall-degrading enzymes in soft rot bacteria. Pectin is degraded by pectin lyases (Pnl). Pectin methylesterases (Pem) remove methyl as methanol from methylated pectin. Unmethylated pectin is targeted by oligogalacturonases (Peh) and pectate lyases (Pel). Xylanases (Xyl) target hemicellulose, cellulases (Cel) target cellulose, and proteases (Prt) target proteins in the cell wall. In the figure, PM signifies the plasma membrane.

1.2.3 Strategies of soft rot proteobacteria

Bacterial soft rot is an agriculturally and economically important plant disease that occurs worldwide and affects tuberous staple crops, often during post-harvest conditions (Charkowski, 2009; Ma et al., 2007; Mansfield et al., 2012; Toth et al., 2003). In potato, soft rot commonly occurs in the tubers but may spread through the stem of the plant. When the infection spreads in this manner, the disease is called blackleg.

Blackleg is mainly associated with *Dickeya* species and *Pectobacterium atrosepticum*, although evidence indicates that *P. carotovorum* ssp. *carotovorum* is a

causative agent of blackleg symptoms as well (Czajkowski et al., 2011; Haan et al., 2008; Toth et al., 2003). The disease is usually observed as a rotting lesion in the stem in wet conditions or as yellowing and wilting in dry conditions (Czajkowski et al., 2011; Monson et al., 2013).

Soft rot bacteria are classified as necrotrophs, although the host interactions of many members of soft rot bacteria are quite specific and show a degree of complexity. Prominent soft rot bacteria include the *Dickeya* and *Pectobacterium* genera (Table 1), both formerly part of the *Erwinia* genus, and all of these genera are part of the *Enterobacteriales* family (Czajkowski et al., 2011; Perombelon and Kelman, 1980). There is evidence of host specificity of some soft rot bacteria, such as *Pectobacterium atrosepticum*, which is associated with infection of potato and close solanaceous relatives (Charkowski, 2009; Chatterjee et al., 1995; Collmer et al., 2009; Glasner et al., 2008; Kay and Bonas, 2009; Toth et al., 2006; Toth and Birch, 2005). Thus, one may speculate that plant-microbe compatibility operates for necrotrophs. This compatibility may differ from the widely examined avirulence resistance gene interactions that are crucial for the hemibiotrophic phytopathogens *Xanthomonas* or *Pseudomonas* ssp. However, in general the host range and spread of soft rot bacteria in nature are wide, implying primary reliance on virulence traits that are not specific for certain hosts (Charkowski et al., 2012). The bacteria enter the plants via tissue openings, such as wounds or lenticels, through the roots from the soil, or from nearby infected plants (for example, via the stolons between tubers). Upon entering, the soft rot bacteria may commence infection or persist as a latent infection depending on the environmental conditions (Czajkowski et al., 2011; Perombelon and Kelman, 1980; Toth et al., 2003). During favorable conditions, the soft rot bacteria begin releasing PCWDEs, causing the maceration of plant tissues and forming a watery mash unfit for human consumption. This mash of rotted tissue may also spread the infection to neighboring tubers in storage facilities (Czajkowski et al., 2011; Toth et al., 2006, 2003). Outside of the host, the bacteria live in soil but do not generally survive for more than 1-6 months. Soft rot bacteria are not known to overwinter in the soil, but they may persist mainly in diseased plants, insects or aerosols during cold seasons (Czajkowski et al., 2011; Toth et al., 2006).

Table 1. Examples of proteobacteria associated with soft rot in various host plants.

Species	Example of host	Reference
Dickeya chrysanthemi	Potato	(Marrero et al., 2013)
Dickeya dadantii	Potato	(Ma et al., 2007)
Dickeya solani	Potato	(Adriaenssens et al., 2012)
Pectobacterium atrosepticum	Potato	(Ma et al., 2007)
Pectobacterium betavasculorum	Sugar beet	(Avrova et al., 2002)
Pectobacterium brasiliensis	Potato	(Ma et al., 2007)
Pectobacterium carotovorum	Potato	(Ma et al., 2007)
Pectobacterium wasabiae	Horseradish	(Avrova et al., 2002)
Pseudomonas cichorii	Lettuce	(Kiba et al., 2006)
Pseudomonas mariginalis	Potato	(Liao et al., 1997)
Pseudomonas viridiflavia	Lettuce	(Liao et al., 1988)

1.2.4 *Pectobacterium wasabiae* strain SCC3193

P. wasabiae is a globally occurring soft rot bacterium, and the name was originally given to a strain isolated from horseradish in Japan (Goto and Matsumoto, 1987). The species strain *P. wasabiae* SCC3193 was isolated from potato in Finland during the 1980s (Pirhonen and Palva, 1988). Recently, *P. wasabiae* was identified in Europe as a common soft rot-causing bacterium (Pasanen et al., 2013). Similar to other soft rot bacteria, *P. wasabiae* is a Gram-negative rod with peritrichous flagella (Charkowski et al., 2012; Czajkowski et al., 2011; Toth et al., 2006). Standard techniques for genetic research that are applicable for basic model organisms, such as *Escherichia coli* and *Salmonella enterica,* are also applicable for *P. wasabiae*, making it a suitable candidate as a model organism for the research of soft rot pathogenesis (Pirhonen et al., 1991; Toth et al., 2006). However, the growth optimum of SCC3193 is 28°C, not 37°C, as that of *E. coli* or *S. enterica* (Perombelon and Kelman, 1980; Pirhonen et al., 1991). Members of the *Pectobacterium* genus, such as *P. wasabiae*, *P. carotovorum* ssp. *carotovorum* and *P. atroseptcium,* were originally placed under the *Erwinia* genus but have recently adopted new names, which is important to consider when reviewing older articles regarding the same organisms (Chatterjee et al., 1995; Marits et al., 2002; Mukherjee et al., 1996; Toth et al., 2006). In this study, the *Pectobacterium* strain SCC3193 is referred to as *P. wasabiae*, as introduced by Nykyri et al., 2012 (Toth et al., 2003). The SCC3193 strain has been used by various research groups globally, mainly in studies concerning PCWDE activity, stress (starvation and high-density population), virulence regulator proteins, quorum sensing (QS), and the *expSA-rsmB-rsmA* (*exp-rsm*) gene regulation system (Andersson et al., 1999, 2000; R. A. Andersson et al., 1999; Barras et al., 1994; Chatterjee et al., 1995; Eriksson et al., 1998; Flego et al., 1997; Marits et al., 2002, 1999; Mukherjee et al., 1996; Pirhonen et al., 1993; Suh et al., 1999).

1.3 General aspects of gene regulation in soft rot bacteria

1.3.1 Regulatory systems

Regulatory systems and networks in γ-proteobacterial phytopathogens seem to rely primarily on QS, sigma factors, regulatory RNA, RNA-interacting proteins, and regulatory two-component systems (TCS). TCS involve transcriptional regulation by transcription factors (TFs) (Mole et al., 2007). Evidence indicates that bacteria in specialized stable niches require relatively few and simple regulatory systems, whereas bacteria in unstable communal environments, such as phytopathogens that live in soil, plants or insect vectors, require more complex regulatory systems (Cases and de Lorenzo, 2005). Changes in the environment, such as fluctuations in available nutrients and temperature, and the interactions between neighboring cells cause bacteria to utilize intricate regulatory systems to adapt. These

adaptations include the release of extracellular compounds to signal nearby cells (Lotze et al., 2007). One example of regulatory adaptations is quorum sensing (QS), which is used to organize the coordination of bacterial communities both within and between species (Barnard et al., 2007; Miller and Bassler, 2001). QS is a cell density-dependent form of communication between cells, and it is present among both Gram-positive and Gram-negative bacteria (Bassler, 1999). The communication is generally based on a concentration gradient of molecules called autoinducers (Miller and Bassler, 2001; Tahrioui et al., 2013). Among Gram-negative bacteria, the most common and well-characterized autoinducers are *N*-acylhomoserine-lactones or homoserine-lactones (AHL and HSL), which are fatty acid-based molecules with varying chain lengths (Andersson et al., 2000; Bassler, 1999; Jimenez et al., 2012; Toth et al., 2006). AHLs and HSLs are generally synthesized by LuxI homologs in proteobacteria and bind to LuxR-type receptors that proceed to modulate gene expression (Burr et al., 2006; Kõiv and Mäe, 2001; Liu et al., 2008). As the bacterial population increases in concentration in an environment, so does the relative concentration of the signaling molecules. The autoinducers are sensed due to diffusion through the cell wall into the neighboring cells (Fuqua et al., 2001). Once the intracellular concentration of the signaling molecule rises above a certain threshold, the activity of bacterial virulence-associated genes is induced, including the genes encoding PCWDEs and proteins for the export of virulence factors in many proteobacterial animal and plant pathogens (Andersson et al., 2000; Bassler, 1999; Charkowski, 2009; Miller and Bassler, 2001).

TCS signaling relies on the interactions between two proteins. One protein is a membrane-associated signal receptor histidine kinase that autophosphorylates a specific intracellular response regulator upon receiving a signal (Mole et al., 2007; Tahrioui et al., 2013; Yang et al., 2008). This response regulator is a TF, which may bind DNA and alter gene expression (Browning and Busby, 2004; Yang et al., 2008). One example of a TCS is ExpS-ExpA in *P. wasabiae* SCC3193 (Hyytiäinen et al., 2001). Sigma factors, on the other hand, are also DNA-binding proteins that serve to directly connect the core RNA polymerase and the promoter sequence of a gene, allowing the assembled protein complex to begin transcription (Browning and Busby, 2004; Helmann and Chamberlin, 1988). Thus, sigma factors are transcription initiation factors (Mole et al., 2007). The post-transcriptional regulation of gene expression includes RNA-binding proteins, non-coding RNAs and small RNAs that interact with transcripts and modulate their integrity in a positive or negative fashion (Romeo et al., 2013).

1.3.2 Transcriptional regulation

The transcriptome is an important target for regulation in cells. Modulation of the transcriptome allows for relatively rapid adjustments to changing environments, compared with modulations of the genome (Mole et al., 2007). The transcriptome may be viewed as the mid-point of the information flow between the genome and proteome or between the DNA blueprints and the protein end products. Transcription can be regulated before or after RNA synthesis (Browning and Busby, 2004). Pre-transcriptional regulation is associated with

transcription initiation factors, such as the sigma factors (Browning and Busby, 2004; Janga et al., 2009; Timmermans and Melderen, 2010). Sigma factors bind the RNA polymerase and direct it to a promoter or operator that is coupled to a gene. The interaction between sigma factor and RNA polymerase modulates transcription initiation (Filloux, 2012; Janga et al., 2009; Thieffry et al., 1998). Different sigma factors have distinct affinities for DNA sequences and can compete with each other for binding to RNA polymerase. This competition between sigma factors can affect the initiation of transcription of distinct genes (Potvin et al., 2008; Timmermans and Melderen, 2010). The sigma factors can be distinguished from the TFs, which do not directly bind RNA polymerase. The primary mechanism of TFs is to interfere with the ability of RNA polymerase to bind to the DNA by blocking promoters or facilitating DNA binding (Filloux, 2012). Thus, TFs may induce or repress the expression of a gene. Normally, TFs target specific DNA sequences and motifs and bind them in a "facilitated diffusion" manner without requiring energy (Browning and Busby, 2004; Janga et al., 2009). The activity of TFs may in turn be regulated by ligands, such as metabolites, or via covalent modification by other proteins (Browning and Busby, 2004). Examples of systems utilizing DNA-binding TFs for regulation include the LacI protein regulating the *lac* operon and GacA, which is a homolog of ExpA (Francke et al., 2008; Hassan et al., 2010; Janga et al., 2009). LacI is a DNA-binding protein that inhibits transcription initiation by RNA polymerase, rending it unable to move out of the *lac* operon promoter region. LacI is in turn shut down by lactose derivatives, such as allolactose (Ozbudak et al., 2004). GacA ties together pre- and post-transcription regulation via the regulation of the expression of ncRNAs, which are RNA molecules that do not encode a protein product. However, ncRNAs may modulate the activity of RNA-binding post-transcriptional regulator proteins (Hassan et al., 2010).

Once the RNA has been synthesized, the molecule may still be directly modified. Such post-transcriptional regulation can be performed by RNA-binding proteins and ncRNAs (González et al., 2008). Certain ncRNAs can interact with mRNAs (in either a trans- or cis-dependent manner) and affect their stability positively or negatively, as well as modulate the activity of RNA-binding proteins (Caldelari et al., 2013; González et al., 2008; Lapouge et al., 2008). RNA-binding regulatory proteins may function in a similar fashion by binding RNA molecules and affecting their stability positively or negatively (Babitzke and Romeo, 2007; Timmermans and Melderen, 2010; Wei et al., 2001). Two systems that exemplify protein-based post-transcriptional regulation are the Hfq (**H**ost **f**actor required for phage **Q**β replication) and CsrA (**c**arbon **s**torage **r**egulator A) systems (Brennan and Link, 2007; Wei et al., 2001). The RNA binding chaperon Hfq aids sRNAs to bind mRNAs. The interaction between Hfq, ncRNAs, and mRNAs affects their stability, putatively via the recruitment of RNAses (Storz et al., 2011; Vanderpool et al., 2011). The CsrA protein binds RNA and affects RNA stability positively or negatively. The protein activity can be further modulated by ncRNAs that interfere with the normal mRNA-binding actions of CsrA (Brencic and Lory, 2009; Hassan et al., 2010; Lapouge et al., 2008). In conclusion, bacteria utilize a variety of overlapping systems to control gene expression (Figure 2).

Other examples of RNA-based transcriptional regulation appear at the interface between transcription and translation, where regulatory sRNAs may bind and inhibit the formation of secondary structures that may hinder translation (Storz et al., 2011).

Additionally, the ligand-binding 5' ends of some mRNAs are known as "riboswitches" (Romeo et al., 2013; Waters and Storz, 2009).

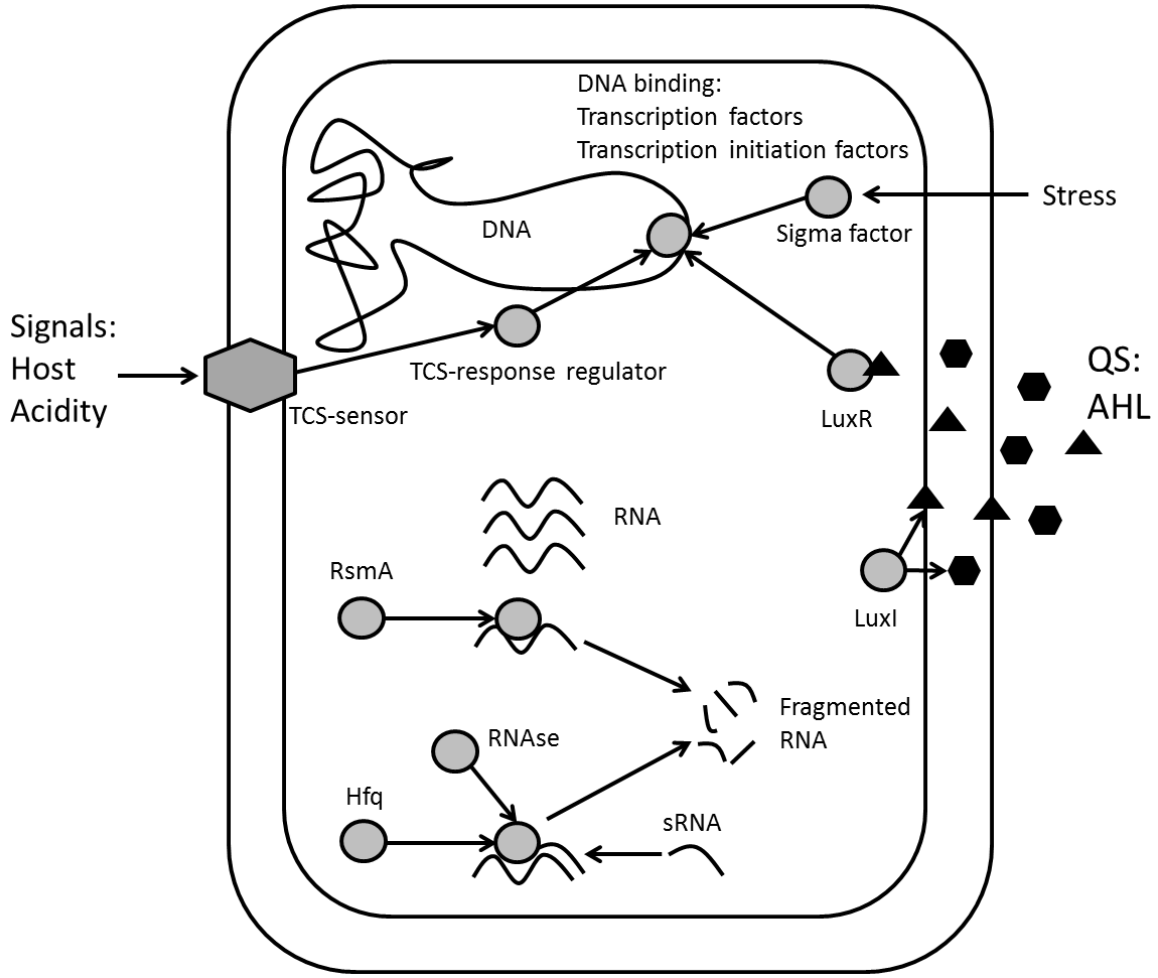


Figure 2. General overview of common regulatory systems in enterobacterial phytopathogens. Two component systems (TCS), quorum sensing (QS) and transcription initiation factors (for example, sigma factors) all bind DNA and serve to regulate gene expression. The signals that activate these systems may originate from bacteria as acyl homoserine lactones (for example, AHL) or from other environmental sources (TCS, sigma factors). Once RNA has been transcribed, further regulation is available via RNA-binding proteins (for example, RsmA or Hfq) or via regulatory small RNAs (sRNAs). Double-stranded RNA (for example, mRNA and sRNA) can be targeted by RNAses and degraded.

1.4 The post-transcriptional regulator RsmA

1.4.1 RsmA is a conserved homolog of CsrA

RsmA is a homolog of CsrA, which was first characterized in *E. coli* as an important inducer of glycogen metabolism (Cui et al., 1995; Romeo et al., 2013, 1993; Whitehead et al., 2002).

The conserved structure of RsmA consists of two monomers, each consisting of 61 amino acids that are structured into five β-sheets and one α-helix (Babitzke and Romeo, 2007; Romeo et al., 1993). By binding to transcripts, RsmA affects their stability in a positive or negative manner (Andrade et al., 2014; Baker et al., 2002; Lapouge et al., 2008; Lawhon et al., 2003; Timmermans and Melderen, 2010; Wei et al., 2001). CsrA homologs have been detected not only among γ-proteobacteria (Figure 3) but also among α-proteobacteria and Gram-positive bacteria (White et al., 1996). For homologs in bacteria, such as *P. syringae*, *P. aeruginosa*, *P. fluorescens*, *P. carotovorum*, and *P. wasabiae*, the nucleotide binding motif of CsrA is GGA (Baker et al., 2007; Brencic and Lory, 2009; Cubitt et al., 2013; Lapouge et al., 2008, 2007; Liu et al., 1998). This motif is usually located in the untranslated 5' leader sequence, or it may overlap with the Shine-Dalgarno sequence near the translation initiation site on the mRNA (Babitzke and Romeo, 2007; Cubitt et al., 2013; González et al., 2008). In the Gram-positive bacterium *Bacillus subtilis*, a homolog of RsmA has been demonstrated to bind RNA and block ribosome binding (Yakhnin et al., 2007). The protein forms a central node for three integral global regulatory networks: quorum sensing (associated with the regulators ExpR and ExpI), stress (for example, RpoS), and regulators suggested to balance the effects of QS and stress on global gene expression (for example, GacA). These regulatory systems are all important for the adaptation of many γ-proteobacteria to their environment (Beier and Gross, 2006; Lapouge et al., 2008; Mole et al., 2007; Santander et al., 2014; Tahrioui et al., 2013).

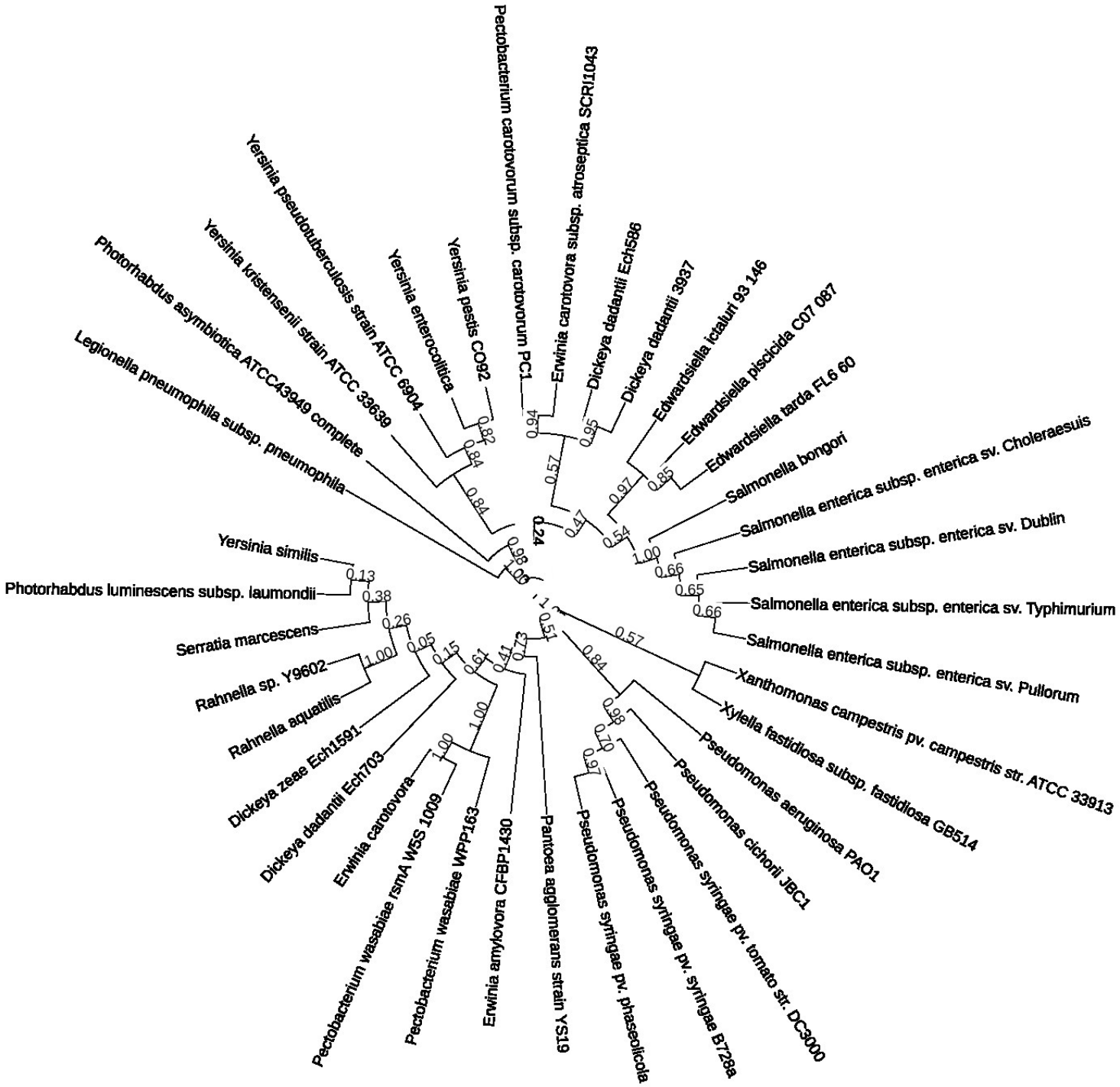


Figure 3. Phylogenetic tree of nucleotide sequence relationships between the *rsmA* gene of *P. wasabiae* and 36 selected animal and plant pathogens. Sequences of *rsmA* homologs were aligned using the Clustal Omega Pearson format (Sievers et al., 2011; Sievers and Higgins, 2014). MEGA6 software was used to construct the maximum likelihood consensus tree from the aligned sequences (Tamura et al., 2013). The consensus tree was visualized using iTOL (Letunic and Bork, 2011). The numeric values in the figure depict bootstrap values calculated from 1000 bootstrap trees.

1.4.2 RsmA regulates virulence in many γ-proteobacteria

RsmA and its homologs seem to play varying but central roles in global gene regulation in plant and animal pathogenic bacteria. A number of studies have revealed an inconsistent list of physiological and virulence-related phenotypes of *rsmA* mutants among γ-proteobacterial plant pathogens (Forsbach-Birk et al., 2004; Kim et al., 2012; Lapouge et al., 2008). In *Pectobacterium* species, RsmA was identified as a negative regulator of many virulence-associated factors, such as PCWDEs, secretion systems, flagellar biosynthesis and motility (Chatterjee et al., 2002, 1995; Monson et al., 2013). The findings regarding *Pectobacterium* agree with some of the observations of *Legionella pneumonphila csrA* knock-down mutants or conditional null mutations. *L. pneumonphila csrA* mutants demonstrate hyperflagellation along with enhanced infectivity and persistence in macrophages and the amoeba *Acanthamoeba castellani* (Forsbach-Birk et al., 2004; Molofsky and Swanson, 2003). Other evidence suggests that RsmA positively and negatively regulates a vast array of virulence-associated genes in the animal and plant pathogen *P. aeruginosa* (Brencic and Lory, 2009; Burrowes et al., 2006; Pessi et al., 2001). Data suggest that a *P. aeruginosa rsmA* mutant exhibits a lowered ability to colonize and spread in mouse models during the initial infection of acute pneumonia. However, a lowered mortality rate in mouse models of chronic infection that were inoculated with *P. aeruginosa csrA* mutants was also observed (Mulcahy et al., 2008). Despite the observed lower mortality rate in the models of chronic infection that were infected with the *rsmA* mutant, the mutant was more successful than the wild-type bacterium in chronic persistence and pulmonary inflammation in the mice (Mulcahy et al., 2008). The effects of an RsmA mutation in the biocontrol bacterium *P. fluorescens* are not apparent unless the homologous allele RsmE is mutated as well, leading to severely increased expression of genes encoding hydrogen cyanide synthase, exoprotease and antiphytopathogenic compound biosynthesis proteins (Lapouge et al., 2008; Reimmann et al., 2005). Conversely, overexpression of RsmA in *P. fluorescens* has been linked to the enhanced production of HCN and exoprotease (Heeb et al., 2002).

The presence of RsmA is important for the functional virulence and positive regulation of extracellular polysaccharides, HR-inducing ability and PCWDEs in the bacteria *Xanthomonas campestris* and *Xanthomonas oryzae* pv. *oryzae* (Chao et al., 2008; Zhu et al., 2011). Furthermore, *rsmA* mutants of the animal pathogen *Salmonella enterica* sv. Typhimurium demonstrate impaired ability to invade epithelial cells, as well as lowered expression of genes involved in host invasion (Altier et al., 2000; Lawhon et al., 2003). In contrast to *P. carotovorum*, *rsmA* mutants in the species *X. campestris*, *X. oryzae*, *S. enterica* sv. Typhimurium and *P. aeruginosa* all demonstrate lowered expression of type 3 secretion system (T3SS)-related genes (Chao et al., 2008; Mulcahy et al., 2006). The T3SS is an important virulence factor for many soft rot enterobacteria and serves as a primary means of transporting effectors to target host cells (Khokhani et al., 2013; Yang et al., 2008). In conclusion, RsmA is an important regulator that is present in animal and plant pathogens. See Table 2 for a brief summary of the impact of RsmA homologs on virulence among phytopathogens.

Table 2. Overview of the importance of RsmA homologs on various virulence-associated phenotypes of selected phytopathogens (Chao et al., 2008; Cui et al., 1995; Heurlier et al., 2004; Kong et al., 2012; Lu et al., 2012; Mukherjee et al., 1996; Mulcahy et al., 2006; Pessi et al., 2001; Zhu et al., 2011).

Bacterial species	Phenotypes*
Dickeya dadantii	(Overexpression) reduction of plant tissue maceration; reduced production of indigoidine pigment, HSL, Cel and Prt.
Erwinia amylovora	(Overexpression) reduction of motility, EPS production, HR-elicitation, virulence.
Erwinia herbicola	(Overexpression) reduced production of EPS, carotenoid pigment.
Pantoea stewartii	(Overexpression) reduced production of EPS, carotenoid pigment.
Pectobacerium carotovorum	(Overexpression) reduction of HR elicitation, virulence, plant tissue maceration; reduced production of EPS, HSL, Cel, Peh and Prt.
Pectobacterium atrosepticum	(Overexpression) reduced plant tissue maceration; reduced production of HSL, Cel, Peh and Prt.
Pectobacterium betavasculorum	(Overexpression) reduced plant tissue maceration; reduced production of HSL, antibiotics, Cel, Peh.
Pectobacterium wasabiae	(Overexpression) reduction of flagella; reduced production of Cel, Peh, Pel and Prt.
Pseudomonas aeruginosa	(Knockout) enhanced antibiotic resistance, chronic persistence in pulmonary infection, airway epithelial cell invasion. Enhanced production of elastase, AHL, HCN, pyocyanin, lectin and Prt. Reduction of staphylolytic activity, cytotoxicity, swarming motility, colonization during initial acute infection, actin depolymerization capacity. Reduced production of effectors, rhamnolipids, lipase and translocation proteins. (Overexpression) reduced production of elastase, Prt, HCN, AHL.
Pseudomonas syringae pv. *syringae*	(Overexpression) reduction of virulence and swarming. Reduced production of phaseolotoxin, alginate, syringomycin, tabtoxin, Prt, pyoverdin and biofilm.
Pseudomonas syringae pv. *phaseolicola*	(Overexpression) reduction of virulence and swarming. Reduced production of phaseolotoxin, syringomycin, tabtoxin, Prt and pyoverdin.
Xanthomonas campestris pv. *campestris*	(Knockout) reduction of swimming motility, virulence, propagation *in planta*; reduced production of Cel, Prt, amylase and EPS. Enhancement of intracellular glycogen accumulation, cell aggregation and adhesion.
Xanthomonas oryzae pv. *oryzae*	(Knockout) reduction in virulence and ability to induce HR. Lower production of diffusible signal factor, EPS, extracellular endoglucanase and extracellular amylase. Higher production of glycogen and biofilm. Enhanced cellular adhesion.

Xylella fastidiosa	(Knockout) enhancement of biofilm production.

* Type of mutation for given phenotypes is in parenthesis.

1.5 ExpA is a global transcriptional regulator in phytopathogens

1.5.1 ExpS and ExpA form a two-component signaling system

Similar to RsmA, ExpA (GacA) is a conserved regulator among many γ-proteobacteria (Figure 4). The protein consists of 221 amino acids and forms a signal TCS with the receptor kinase ExpS (Frederick et al., 1997; Hyytiäinen et al., 2001; Storz et al., 2011). TCS systems are widely distributed among bacteria and serve as important switches for adaptation to changing environments (Yang et al., 2008). The regulatory function of ExpA and its homologs have been implicated to mainly concern the activity of RsmA in soft rot bacteria (Cubitt et al., 2013; Lapouge et al., 2008). The positive influence of ExpA-mediated regulation on virulence and social interactions for bacteria seems clearer than that of RsmA (Lapouge et al., 2008). Pathogenic bacterial mutants of *expA* homologs generally become avirulent, and in the case of biocontrol strains, such as *P. fluorescens* and *Pseudomonas chlororaphis*, they lose some of their biocontrol abilities (Hassan et al., 2010; Lapouge et al., 2008; Reimmann et al., 1997; Wang et al., 2013). The exact chemical structure of the signal that causes ExpS autophosphorylation and subsequent ExpA activation is currently unknown, although plant-derived phenolic or acidic compounds have been implicated as putative inducers in *P. wasabiae, P. aeruginosa* and *D. dadantii* (Charkowski, 2009; Eriksson et al., 1998; Khokhani et al., 2013; Li et al., 2009; Yamazaki et al., 2012; Yu et al., 2014). Once ExpA is phosphorylated, it may bind DNA sequences and affect the transcription of genes. ExpA homologs have been found to affect the transcription of ncRNAs, and in *Pectobacterium* species, the ncRNA regulated by ExpA is transcribed from a gene called *rsmB* (Chatterjee et al., 2002; Hyytiäinen et al., 2001).

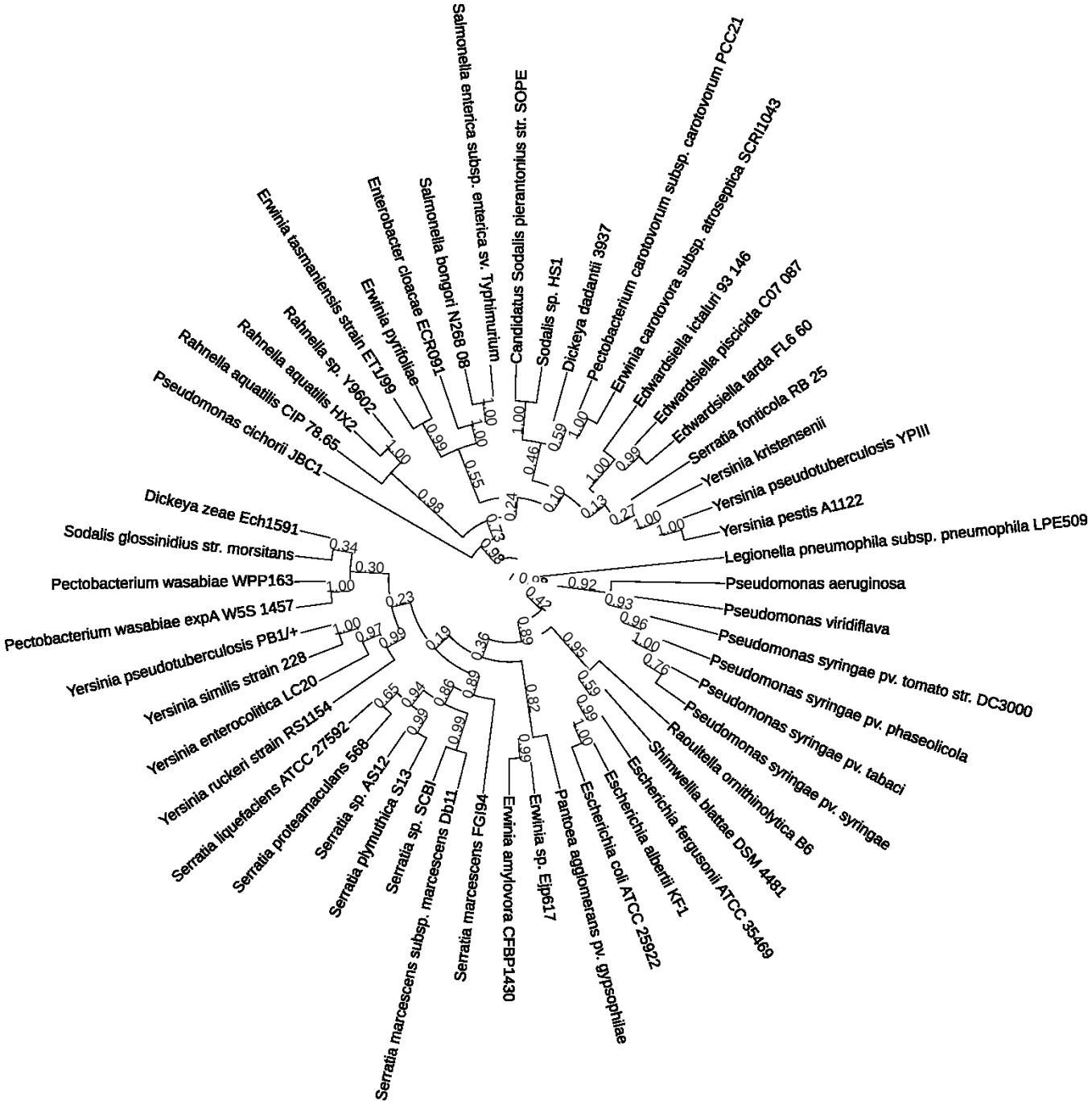


Figure 4. Phylogenetic tree of nucleotide sequence relationships between the *expA* gene of *P. wasabiae* and 51 selected animal and plant pathogens. Sequences of *expA* homologs were aligned using the Clustal Omega Pearson format. MEGA6 software was used to construct the maximum likelihood consensus tree from the aligned sequences. The consensus tree was visualized using iTOL. The numeric values in the figure depict bootstrap values calculated from 1000 bootstrap trees.

1.5.2 ExpA modulates RsmA activity via *rsmB*

A gene encoding the ncRNA that is bound by CsrA and inhibits its activity was originally described in *E. coli* and named *csrB* (Liu et al., 1997). The functionally homologous gene

rsmB in soft rot bacteria encodes a small ncRNA that contains several repeats of the RsmA nucleotide binding motif and stem-loop structures, serving to effectively bind several RsmA proteins. Thus, the binding of RsmA proteins to the transcript of *csrB* hinders those same RsmA proteins from binding to target mRNAs (Cubitt et al., 2013). The *rsmB* gene has been implicated as an important part of the GacA regulation cascade in soft rot bacteria, such as *P. carotovorum*, *E. amylovora, P. atrosepticum*, *D. dadantii* and *E. herbicola* pv. *gypsophilae* (Charkowski, 2009; Cubitt et al., 2013; Cui et al., 2001; Hyytiäinen et al., 2001). Contrary to the general consensus function of RsmA as a destabilizing agent of RNAs, the *rsmB* transcript is stabilized by being bound by RsmA in *Pectobacterium carotovorum* (Chatterjee et al., 2002). This important role of regulating RsmA activity indirectly makes *rsmB* an integral part of the ExpA regulon. This aspect of the ExpA-RsmA regulation seems highly conserved among γ-proteobacteria (Burrowes et al., 2006; Hyytiäinen et al., 2001; Lapouge et al., 2008). In *P. fluorescens* and *P. aeruginosa*, as well as in *Vibrio cholera* and *Salmonella enterica*, there are several *rsmB* homologs in the same genome. The multiple identified genes encoding GacA-regulated ncRNAs suggest a more sophisticated regulation by ExpA homologs in those species (Babitzke and Romeo, 2007). Furthermore, evidence shows that ncRNAs that are unrelated to RsmA or exoprotein regulation are regulated directly or indirectly by ExpA homologs in *P. fluorescens* and *P. aeruginosa*. One example is the transcript of the gene *rgsA* (González et al., 2008). The RNA product of *rsmB* found in *Pectobacterium* demonstrates little direct sequence homology to *csrB* in *E. coli*, as it is approximately 480 bp long and subject to processing into a smaller 250 bp fragment (Liu et al., 1998).

Thus far, research on the regulation performed by ExpA and RsmA in *P. wasabiae* SCC3193 and other phytopathogens has been primarily focused on the motility, virulence and production of various PCWDEs (Figure 5).

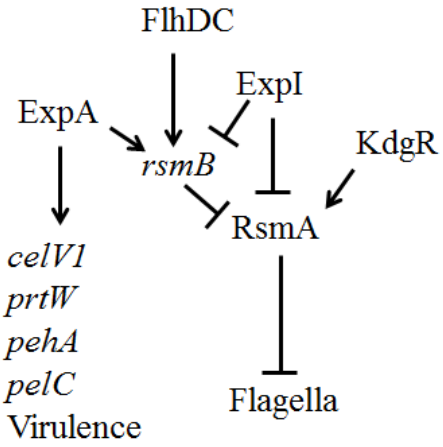


Figure 5. Established regulation of the *exp-rsm* pathway in *P. wasabiae* SCC3193 (Andresen et al., 2010; Eriksson et al., 1998; Hyytiäinen et al., 2001; Kõiv and Mäe, 2001; Mukherjee et al., 1996; Pirhonen et al., 1991). In the figure, arrows indicate positive regulation, whereas truncated lines signify negative regulation.

1.5.3 Regulation via the *exp-rsm* pathway

As mentioned previously, the *exp-rsm* regulators affect social functions of bacteria as well as virulence. Numerous studies have investigated what is regulated by *exp-rsm* homologs in plant pathogenic relatives of *P. wasabiae*. A mutation in *expA* (*gacA* or *repB*) in various phytopathogens (see Table 2 for a summary) generally causes avirulence and reduced production of various virulence determinants (Chatterjee et al., 2003; Mhedbi-Hajri et al., 2011; Shi et al., 2009; Yang et al., 2008). For many bacteria where ExpA is a central regulator of virulence or exoenzyme production, the colony morphology and culture growth rates are usually similar between different wild-type and *expA* mutants (Hassan et al., 2010; Kidarsa et al., 2013; Shi et al., 2009; Yang et al., 2008). Mutations in the homologs of the genes encoding the *expSA* TCS in different plant-pathogenic *Pseudomonas* ssp. result in a decrease in the production of exoenzymes and other virulence factors (Chatterjee et al., 2003; Cui et al., 2001; Hrabak and Willis, 1992; Loh et al., 2002). A study of *Pseudomonas marginalis* found that a mutation in either *gacA* or *lemA* (a *gacS* homolog) causes a decrease in virulence and in the production of Pel and Prt (Liao et al., 1997). However, in that same study, the *gacA* mutant produced less of the iron-scavenging siderophore pyroverdine and the polysaccharide levan than the *lemA* mutant (Liao et al., 1997). The differences observed between *gacA* and *gacS* mutations in *P. marginalis* further suggest that *expA* homologs may have a more significant impact on virulence than *expS* homologs under certain circumstances. In animal pathogens, such as *S. typhimurium,* further evidence has demonstrated that the *expA* homolog *sirA* is essential for virulence and intracellular survival (Ahmer et al., 1999; Chan et al., 2005). In uropathogenic *E. coli*, a mutation in *uvrY* (*expA*) causes a reduction in the ability of the mutant to survive in the urinary tract (Tomenius et al., 2006). The loss of virulence by inactivating an *expA* homolog has also been observed in the animal pathogens *L. pneumophila*, *P. aeruginosa*, *Vibrio cholerae* and *Serratia marcescens* (Hammer et al., 2002; Lapouge et al., 2008; Lenz et al., 2005; Williamson et al., 2006; Wong et al., 1998). Along with auxiliary regulators, such as *rpoS* and QS, the *exp-rsm* system appears to be mainly utilized for the regulation of gene expression during late-logarithmic and stationary phases among proteobacteria (Babitzke and Romeo, 2007; Hassan et al., 2010; Romeo et al., 1993; Tahrioui et al., 2013). Generally, loss-of-function phenotypes have been characterized in studies of *gacA* mutants, whereas gain-of-function phenotypes have been reported to a lesser extent (Lapouge et al., 2008).

Large-scale transcriptomic studies of *exp-rsm* regulation in phytopathogens have been performed on *X. fastidiosa* (*expA*), *X. campestris* (*rsmA*), *P. aeruginosa* (*rsmA*) and *P. atrosepticum* (*rsmB*) (Burrowes et al., 2006; Chao et al., 2008; Cubitt et al., 2013; Shi et al., 2009). In investigations involving *expA*-related regulation, studies affirm the reduced-virulence phenotypes described earlier, where virulence-associated genes were found to be down-regulated in the mutants. These virulence-associated genes include fimbrial adhesion and biofilm formation in *X. fastidiosa* and PCWDEs in *P. atroseptcium*. The transcriptomic studies of *expA* mutants in *P. wasabiae* SCC3193 primarily utilized Northern blot analysis and gene fusion constructs. These studies revealed a reduction in PCWDE-related transcripts

in the *expA* mutants compared with the wild-type, resulting in impaired virulence in the mutants (Eriksson et al., 1998; Hyytiäinen et al., 2001; Marits et al., 2002).

Table 3. Overview of the importance of *expA* and its homologs in selected phytopathogens on various virulence-associated phenotypes (Chatterjee et al., 2010, 2003; Cui et al., 2001; Eriksson et al., 1998; Frangipani et al., 2014; Hyytiäinen et al., 2001; Kay et al., 2006; Kinscherf and Willis, 1999; Lebeau et al., 2008; Liao et al., 1994; Marutani et al., 2008; Panijel et al., 2013; Pirhonen et al., 1991; Reimmann et al., 1997; Rich et al., 1994; Willis et al., 2001; Yang et al., 2008).

Bacterial species	*expA* homolog	Impaired phenotypes
Dickeya dadantii	*gacA*	Virulence; production of Cel, Pel, Prt, biofilm and pellicles.
Erwinia amylovora	*gacA*	Swarming motility; virulence; HR elicitation; production of EPS, harpin and siderophores.
P. aeruginosa	*gacA*	Swarming motility; virulence; resistance to UV; production of AHL, HCN, pyocyanin, pyochelin lipase, elastase, chitinase, antibiotic resistance and biofilm.
Pantoea agglomerans	*gacA*	Virulence and gall formation.
Pectobacerium carotovorum	*gacA*	Cel, Pel, Peh, Prt production
Pectobacterium wasabiae	*expA*	Cel, Peh, Pel, Prt production and virulence
Pseudomonas chicorii	*gacA*	Protease and necrosis inducing factor.
Pseudomonas mariginalis	*gacA*	Virulence; production of levan, pyroverdine, Pel and Prt.
Pseudomonas syringae pv. *syringae*	*gacA*	Swarming; virulence; production of syringomycin, AHL, alginate and Prt.
Pseudomonas syringae pv. tomato	*gacA*	Pigmentation, swarming motility, virulence, propagation *in planta* and HR elicitation.
Pseudomonas viridiflava	*repB*	Virulence; production of siderophores and Pel.
Pseudomonas syringae pv. *tabaci*	*gacA*	Pigmentation, AHL production, swarming motility, virulence, adhesion, propagation *in planta*, HR elicitation and adhesion.
Xylella fastidiosa	*gacA*	Adhesion, cell-to-cell aggregation, biofilm production, virulence.

1.6 Pathogen defense in *Arabidopsis thaliana*

1.6.1 Plant-pathogen interactions

Plants constantly come into contact with bacteria and other microbes, which can originate from the soil, water, air or other plants and animals. Plants interact with potential bacterial pathogens, and these interactions may be categorized as pathogenic, parasitic or mutualistic (Newton et al., 2010). Some of these microbes can cause damage to plants and are thus called phytopathogens. An example of a mutualistic interaction is the relationship between certain

legumes and Gram-negative bacteria of the rhizobia group, where the bacteria perform the fixing of atmospheric nitrogen inside the plants in exchange for nutrients (Long, 1996; Newton et al., 2010). In the case of parasitism, the microbe causes indirect damage to the host by extracting nutrients (Newton et al., 2010). There are few bacterial examples of parasitism, but fungi, such as *Puccinia striiformis* and *Blumeria graminis,* have been classified as strictly biotrophic parasites (Newton et al., 2010; Schulze-Lefert and Panstruga, 2003).

The bacteria *P. wasabiae* and *P. carotovorum*, which were used as pathogen models in this dissertation, are examples of necrotrophic phytopathogens. These two bacterial species require active degradation of host cells for survival and propagation during infection (Czajkowski et al., 2011; Newton et al., 2010). However, soft rot bacteria may not always be constantly virulent when inside plants and can spend part of their life cycle in a more latent or parasitic fashion (Czajkowski et al., 2011). To successfully infect and propagate, the bacteria need to gain access to the plant's interior, such as the apoplast. However, the bacteria cannot themselves penetrate the plant cuticle to access the plant's inner tissues, and thus they require pre-existing openings, such as stomata, before infection can commence (Charkowski et al., 2012). Once conditions are favorable for the bacteria to commence invasion, the more complex interactions between host and pathogen begin to occur. As the bacteria enter their hosts and infection may begin, plants in turn utilize various receptors to identify the invaders and release antimicrobial chemicals and proteins to remove the threat. Other responses, such as cell wall strengthening, localized programmed cell death (PCD) and the induction of phytohormonal pathways, are triggered by the infection. Plant defenses can subsequently branch into more specialized responses, depending on the type of pathogen they are exposed to and how the pathogen affects the basal defense responses.

1.6.2 Preformed and inducible plant defense

Before the infection can commence, bacterial phytopathogens first need to overcome the preformed defense of plants. Preformed defenses consist in part of the structural barriers, for example, the epidermis of plants (for example, the epicuticular wax on leaves) and trichomes. Furthermore, constitutively present toxic antimicrobial chemicals, such as saponins and glucosinolates, can sometimes be considered preformed defenses (Holt III et al., 2003; Raacke et al., 2006; Shangguan et al., 2008; van Loon et al., 2006; Wittstock and Gershenzon, 2002; Xiao et al., 2004). The target of phytopathogenic γ-proteobacteria is normally the apoplast. For example, *P. syringae* is mainly associated with host entry via stomata but may also invade via wounds and lenticels. In contrast, soft rot pathogens, such as *Pectobacterium* species, are mainly associated with plant entry via wounds and lenticels but may also access the plant interior via stomata (Buell et al., 2003; Czajkowski et al., 2011; Jones and Dangl, 2006; Perombelon and Kelman, 1980; Smadja et al., 2004). Once γ-proteobacterial phytopathogens have gained access to the apoplast, they may begin attacking and colonizing the plant tissues (Alfano and Collmer, 1996).

Soft rot bacteria use a variety of toxins and PCWDEs to degrade plant cells, sometimes in combination with effector proteins that manipulate defensive plant responses.

As the bacterial mechanisms for invasion have evolved varying routes for circumventing plant defenses, the same has occurred in plant pathways for microbial recognition and the suppression of infection (Baker et al., 1997; Chisholm et al., 2006). As the bacterial invasion commences, receptors in the plasma membrane of plant cells are exposed to pathogen-associated molecular patterns (PAMPs). Examples of PAMPs are fungal chitin, bacterial elongation factors or flagellin (Chisholm et al., 2006; Daudi et al., 2012; Zvereva and Pooggin, 2012). The inducible plant resistance to pathogens is commonly referred to as innate immunity and consists mainly of PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI) (Boller and He, 2009; Jones and Dangl, 2006; Pastor et al., 2013; Pieterse et al., 2009; Thomma et al., 2011).

PAMP receptors are primarily receptor-like kinases that initiate a PTI response upon the recognition of PAMPs (Chisholm et al., 2006; Zhang et al., 2010; Zvereva and Pooggin, 2012). PTI responses include general antimicrobial strategies, such as callose deposition for strengthening damaged cell walls, closing stomata to hinder additional invaders, and the production of reactive oxygen species (ROS), such as superoxide and hydrogen peroxide, which can participate in defense signaling as well as directly damage invaders (Bolwell and Wojtaszek, 1997; Melotto et al., 2006; Nürnberger and Lipka, 2005; Pastor et al., 2013). Additional antimicrobial compounds and metabolites that are produced by plants (as preformed or inducible defense) include nitric oxide (NO) and phytoalexins such as camalexin (Clay et al., 2009; Denoux et al., 2008; Schlaeppi et al., 2010). These defenses are often coordinated and induced via calcium-dependent and mitogen-activated protein kinase (CDPK and MAPK) phosphorylation cascades, phytohormone synthesis and regulators of gene expression (Daudi et al., 2012; Jones and Dangl, 2006; Zvereva and Pooggin, 2012). PTI is commonly regarded as a part of the basal defense of plants, but it is unable to hinder pathogenesis in compatible host plants (Nürnberger and Lipka, 2005).

As a result of the ongoing evolution of plant defense, many pathogens have developed sophisticated arsenals of host-interacting proteins to hinder and circumvent the basal plant defenses. Among the most widely studied PTI avoidance mechanisms utilized by soft rot or hemibiotrophic bacteria are the proteins known as effectors, which are primarily associated with the T3SS, T4SS and T6SS (Alvarez-Martinez and Christie, 2009; Chisholm et al., 2006; Espinosa and Alfano, 2004; Salomon et al., 2014; Toth et al., 2006). T3SS-related effectors have mainly been studied in phytopathogens, such as *P. syringae* and *X. campestris*, and they have been observed to interfere with various aspects of PTI defense responses, such as MAPK cascades or the FLS2 receptor (Block et al., 2008; Chisholm et al., 2006; Jones and Dangl, 2006; Lindeberg et al., 2012; Zvereva and Pooggin, 2012). Effectors and genes encoding effector-like products have also been identified in the soft rot pathogen *P. carotovorum*. However, the effectors of soft rot bacteria are relatively poorly understood, although two such proteins called HrpN and DspE/A have been identified and characterized to be involved in the manipulation of PTI (Cui et al., 2008; Toth and Birch, 2005).

As a response to the effectors produced by bacteria, plants have sequentially evolved ETI. ETI relies on effector-recognizing resistance (R-) proteins (Jones and Dangl, 2006; Zhang et al., 2010). Effectors recognized (directly or indirectly) by R-proteins of non-host plants are commonly termed avirulence (Avr-) proteins (Jones and Dangl, 2006; Zvereva and Pooggin, 2012). The ETI response is a more powerful and swift version of PTI and results in

effects, such as enhanced ROS and antimicrobial metabolite production. Effector-triggered programmed cell death (PCD), also known as the hypersensitive response (HR), is a component of ETI. The HR may quickly hinder infection by biotrophic and hemibiotrophic pathogens, as they require living cells for successful infection (Jones and Dangl, 2006; Klement and Goodman, 1967; Zvereva and Pooggin, 2012). The HR is usually restricted to infected cells, causes localized cell death at the infection site, and hinders further spread of the pathogens (Baker et al., 1997; Jones and Dangl, 2006). One effect associated with the induction of the HR is the activation of systemic acquired resistance (SAR), an SA-dependent non-local general enhancement of the defenses throughout the plant (Baker et al., 1997). In general, ETI is what determines whether a plant-pathogen interaction is compatible (successful infection by the pathogen due to a lack of ETI and ineffective PTI and preformed defense), incompatible (successful resistance by the plant via deployment of ETI, PTI, and the preformed defenses) or non-host (unsuccessful infection by the pathogen due to not being adapted to that particular plant species/cultivar), which are common terms used to describe these interactions (Zhang et al., 2010). The interchange of invading pathogen using Avr-proteins and plant defense with R-proteins is described in the so-called zigzag model (Jones and Dangl, 2006; Nürnberger and Lipka, 2005).

1.6.3 Roles of phytohormones in plant defense

When the plant recognizes PAMPs, a more long-term or even systemic defense response may be induced. This response relies in part on the signaling by various phytohormones (Pieterse et al., 2009). Salicylic acid (SA), jasmonic acid (JA), abscisic acid (ABA), and ethylene (ET) are hormones that plants require to effectively activate the corresponding signaling pathways that are associated with pathogen resistance (Conrath, 2011). The involvement of all of these hormones has been observed in signaling during the PTI and ETI (Tsuda and Katagiri, 2010). Generally, SA-regulated defense is associated with biotrophic pathogens and is regarded as a trigger of SAR, whereas JA and ET are associated with the response against necrotrophic pathogens (Anderson et al., 2004; Pieterse et al., 2009).

SA usually acts in an inhibitory manner toward resistance mediated by JA and ET, and vice versa. Furthermore, studies have suggested the interdependence of JA and ET in the pathogen response (Glazebrook et al., 2003; Penninckx et al., 1998). However, gene expression studies have revealed a large overlap between genes activated by SA and JA, signifying an overall common response against pathogens irrespective of whether they are biotrophic or necrotrophic (Katagiri, 2004). Similar crosstalk as that observed between defense-related hormones also exists between hormones involved in development and defense. For example, the phytohormone ABA is involved in leaf and bud development, and it has been reported as an antagonist of JA and ET (Anderson et al., 2004; Koornneef et al., 1989; Skriver and Mundy, 1990). Additionally, ABA seems to modulate the balance between hormonal signals relating to abiotic and biotic stress, possibly also between defense and plant development (Anderson et al., 2004). In general, one may expect that a necrotrophic pathogen, such as *P. wasabiae,* would be associated with the activation of JA- and ET-associated defense pathways.

1.6.4 Signaling triggered by damage-associated molecular patterns

In addition to PAMP-mediated signaling, plants also have receptors for signs of indigenous molecules signifying internal damage (Krol et al., 2010; Lotze et al., 2007; Ranf et al., 2011). These molecules are called damage-associated molecular patterns (DAMPs), which may induce defense responses, such as PTI, similar to PAMPs (Figure 6) (Chisholm et al., 2006; Lotze et al., 2007; Pastor et al., 2013). Furthermore, DAMP and PAMP signaling often confer membrane depolarization of plant cells by fluctuations in the cytosolic anion and Ca^{2+} concentrations (Krol et al., 2010; Ranf et al., 2011). Characterized examples of DAMPs in plants include the peptide AtPep1 and extracellular ATP (Choi et al., 2014; Krol et al., 2010). AtPep1 provides a contrast with its two receptors PEPR1 and PEPR2 to PAMPs such as Flg22 (a fragment of the flagellin protein) and Elf18 (a fragment of the bacterial elongation factor Tu protein), which are recognized by the corresponding receptors FLS2 and EFR. The inactivation of these receptors causes insensitivity to the PAMPs Flg22 and Elf18, respectively (Krol et al., 2010; Yamaguchi et al., 2010). In constrast, inactivation of PEPR1 or PEPR2 only causes partial insensitivity to AtPep1 elicitation (Yamaguchi et al., 2010).

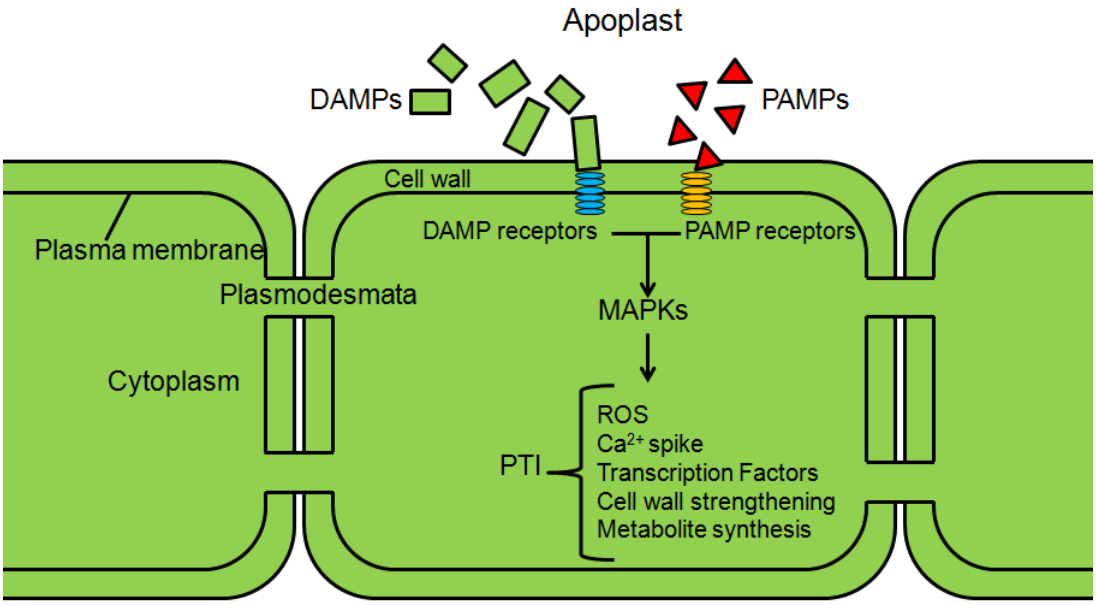


Figure 6. General overview of plant cell responses to damage/pathogen-associated molecular patterns (DAMPs/PAMPs). Specific receptors are activated by binding their respective target DAMP/PAMPs and subsequently transfer the signal downstream via mitogen-activated protein kinases (MAPKs), for example. The MAPKs in turn activate various defense responses involved in the PAMP-triggered immunity (PTI), such as the production of reactive oxygen species (ROS), Ca^{2+} release, antimicrobial metabolite synthesis and the activation of transcription factors for further activation of defense-related genes.

One important type of DAMP-associated elicitors is the oligogalacturonides (OGs), which are oligosaccharides released from the plant cell wall upon pectin degradation by polygalacturonases secreted by pathogens, such as *P. wasabiae* (Denoux et al., 2008; Ferrari et al., 2007; Galletti et al., 2011; Moscatiello et al., 2006). OGs have been implicated as DAMPs due to their promotion of ROS and callose accumulation upon recognition (Ferrari et al., 2013). The membrane protein WAK1 has been identified as a functional receptor of oligogalacturonides (Brutus et al., 2010). The OGs can be of different lengths, depending on the mechanisms of distinct types of digestive enzymes. In general, long OGs (OGs with a degree of polymerization (DP) >10) have been considered biologically active DAMPs in *A. thaliana* (Ferrari et al., 2007; Rasul et al., 2012; Ridley et al., 2001). However, previous studies have shown that shorter OGs may induce the expression of genes involved in JA signaling in *A. thaliana*, enhance resistance against *P. atrosepticum* in potato, and elicit ET production associated with the expression of a gene involved in ET biosynthesis in tomato (Norman et al., 1999; Simpson et al., 1998; Weber et al., 1996). The OG-induced pathway of DAMP signaling has been reported to be independent of common defense-related signaling pathways, for example, those involved in the synthesis of JA, SA and ET (Ferrari et al., 2013, 2007). The transcriptome of plants treated with long OGs has been explored, and comparative analysis with plants treated with a non-inducing medium revealed that long OG signaling has wide-reaching effects on genes associated with defense and metabolism (Denoux et al., 2008; Ferrari et al., 2007). Furthermore, the same studies found large overlaps in transcriptomic effects in *A. thaliana* between OGs, the PAMP elicitor flg22, and the pathogen *B. cinerea* (Denoux et al., 2008; Ferrari et al., 2007). Another study utilized *A. thaliana* cell cultures treated with OGs, DP between 5 to 15, to demonstrate the OG-related modulation of the expression of resistance-related genes (Moscatiello et al., 2006). The use of PAMP- and DAMP-associated elicitors on healthy plants has been observed to prime the plant's defense and enhance pathogen resistance (Galletti et al., 2011; Pastor et al., 2013). The PAMP/DAMP priming promotes SAR in order for the plant cells to more swiftly respond to lower levels of potential elicitors of real pathogens (Conrath, 2011; Heil, 2001; Pastor et al., 2013).

2 AIMS OF THE PRESENT STUDY

The aim of this study was to clarify the signaling and regulation occurring during the interactions of soft rot bacteria and plants. Genetics and transcriptomics were used as the main research methods. One of the goals was to clarify the regulation by the global virulence regulators ExpA and RsmA in the soft rot bacterium *P. wasabiae*. Another goal of this study was to use the host plant *A. thaliana* to explore the importance of short OGs in damage-associated signaling in plants. This study can be divided into three parts:

1) To characterize important virulence-associated genes and pathways in the genome of SCC3193.

2) To perform comparative transcriptomic and phenotypic analyses of bacterial strains containing mutations in *expA* and/or *rsmA*, which are both associated with virulence regulation.

3) To perform comparative transcriptomic and phenotypic analyses of OG signaling in *A. thaliana*.

3 MATERIALS AND METHODS

Materials, methods and model organisms used in this study are presented in detail in the respective publications (I-IV).

Method	Publication
DNA/RNA extraction and purification	I, II, III, IV
Site-directed mutagenesis	I, III
PCR	I, III, IV
RT-PCR	III, IV
Quantitative RT-PCR	III, IV
Microarray hybridization	II, III
Microarray data analysis	II, III
RNA sequencing data analysis	IV
Comparative transcriptomics	III, IV
Genome browsing using BLAST	I, II, III, IV
Gene cloning with ligation and restriction	III
In planta bacterial growth assays	I, III, IV
Statistical analysis with pairwise and multiple comparisons	I, III, IV
Gene clustering and enrichment analysis	IV
Motility assays	III
Semi-quantitative PCWDE activity assay	III
In vitro growth curves	I, III
Bacterial phosphonate sensitivity assay	III
Plant growth retardation assay	IV
Quantitative ROS production analysis	IV

Organism/model	Publication
Arabidopsis thaliana	I, III, IV
Nicotiana tabacum	I, II, IV
Pectobacterium carotovorum ssp. *carotovorum* SCC1	IV
Pectobacterium wasabiae SCC3193	I, II, III
Solanum tuberosum	I

4 RESULTS AND DISCUSSION

4.1 Genomic features of *Pectobacterium wasabiae* SCC3193 (I, II, III)

Pectobacterium wasabiae strain SCC3193 was isolated from potato in Finland in the 1980s. The bacterium was originally taxonomically placed in the genus *Erwinia*. The strain was later placed in the *Pectobacterium* genus as *Pectobacterium carotovorum* ssp. *carotovorum*. Recently, the strain was re-classified as *P. wasabiae* after sequencing and analysis of the genome (I). Nonetheless, *P. wasabiae* SCC3193 has been used as a model organism in soft rot bacterial research for decades (Andersson et al., 1999; Eriksson et al., 1998; Hyytiäinen et al., 2001; Nykyri et al., 2012; Pirhonen et al., 1991). The genome of *P. wasabiae* SCC3193 was sequenced and annotated, and several striking features were detected: the lack of a T3SS normally found in soft rot bacteria, the presence of two type 6 secretion systems (T6SS), and a novel bacterial microcompartment (I). Furthermore, *P. wasabiae* strain SCC3193 was revealed to be phylogenetically more closely related to *Pectobacterium atrosepticum* than to *P. carotovorum*, based on a comparison of over 50 orthologous protein groups between 53 related bacterial strains and yeasts (I). Additionally, the phylogenetic analysis demonstrated a relatively large gap in the relationship between the genera *Pectobacterium* and *Erwinia*, in which *Pectobacterium* is grouped more closely with *Serratia* and *Yersinia* species. The two T6SS of SCC3193 display some overlapping functions regarding plant virulence but differ in their regulation (I, II).

In total, 39 genes were identified as encoding various types of PCWDEs, including pectin and pectate lyases (Pel)/acetylesterases/methylesterases, proteases (Prt), polygalacturonases (Peh), cellulases (Cel), one oligogalacturonide lyase, one rhamnogalacturonate lyase, and one expansin (I, III). A T2SS that is connected with the secretion of PCWDEs was identified along with a type IV secretion system (T4SS). Currently, the products that the T4SS transports are unknown.

As a result of the sequencing and subsequent annotation, the presence of previously identified virulence regulators were confirmed in *P. wasabiae*. These regulators include two-component systems, such as ExpAS, PehRS, PmrAB. Other types of virulence-associated regulators identified in the sequencing were, for example, ExpRI, RcsBCD, RsmA, KdgR, KdgM, RpoS, HexA, and the ncRNA products of *hor* and *rsmB* (Andersson et al., 1999; Andresen et al., 2007; Eriksson et al., 1998; Hyytiäinen et al., 2003, 2001; Kõiv and Mäe, 2001; Pirhonen et al., 1991). The annotation of the SCC3193 genome allowed a subsequent microarray-based comparative transcriptomic analysis of two of these regulators, RsmA and ExpA (II, III).

4.2 Identifying the RsmA regulon in *P. wasabiae* (II, III)

4.2.1 RsmA substantially influences central functions of *P. wasabiae*

The regulatory role of RsmA was previously characterized by large-scale transcriptomic analysis in the phytopathogens *Xanthomonas campestris* and *Pseudomonas aeruginosa* (Brencic and Lory, 2009; Burrowes et al., 2006; Chao et al., 2008). To further clarify the regulatory role of RsmA and its homologs among soft rot bacteria, a similar comparative transcriptomic analysis was performed by utilizing *P. wasabiae rsmA* mutants. Two comparative gene expression investigations were performed utilizing wild-type *P. wasabiae* and two different *P. wasabiae rsmA* mutants, one for each investigation: a knockout mutant (*rsmA*$^-$) and a deletion mutant (Δ*rsmA*), respectively (II, III). In the first study, the analyzed bacterial strains were grown on M9 minimal medium plates supplemented with 10% potato tuber extract (II). In that study, the primary interest was the RsmA regulon during conditions similar to those found *in planta*. In the second study, the analyzed bacterial strains were grown in liquid minimal inducing medium supplemented with 0.4% polygalacturonic acid (PGA) as a carbon source (III). In this study, one aim was to explore the regulon of ExpA by comparing it to the RsmA regulon. This type of comparison would contribute to an understanding of the *exp-rsm* system of regulation in phytopathogens such as *P. wasabiae*. To perform this comparison, not only was the *P. wasabiae* wild-type utilized, but *expA* and Δ*rsmA* mutants and a double mutant (DM) harboring mutations in both the *expA* and *rsmA* genes were used. Both studies revealed that a large part of the analyzed transcriptome, over 25% (more than 1000 genes), was significantly affected by an *rsmA* mutation. Virulence-associated genes that were up-regulated in the *rsmA*$^-$ and Δ*rsmA* mutants when compared with the wild-type strain encoded, for example, T2SS, T6SS, PCWDEs, and flagella. The transcriptomic data associated with the production and secretion of PCWDEs was confirmed by RT-qPCR and enzyme activity assays (III). The enzymatic activity assays revealed an increase in Peh and Pel production in the *rsmA*$^-$ mutant in medium containing PGA or glucose as carbon sources in particular during the exponential growth phase (II). Furthermore, the production of Peh and Pel in the *rsmA*$^-$ mutant was not significantly altered during early exponential growth when comparing bacteria cultured in medium containing either of the two different carbon sources. Significant differences between the *P. wasabiae* wild-type and Δ*rsmA* mutant were observed for the activities of Cel and Peh combined with Pel on semi-quantitative plate assays (III).

In addition to controlling genes directly associated with PCWDE production, the results from this study revealed that RsmA has a significant impact on the transcription of genes involved in motility, growth, cell morphology, glycogen metabolism, and fermentation in *P. wasabiae*. In phenotypic tests, the *rsmA* mutants lost the capacity for swimming motility in minimal medium motility plates with low agar concentrations. However, the mutants displayed swarming motility earlier when compared with wild-type on minimal medium supplemented with potato extract (II). Electron microscopy images demonstrated that the *rsmA*$^-$ cells were highly elongated and hyperflagellated when cultured on medium containing potato extract. The importance of motility for virulence has been well characterized for soft rot pathogens, such as *D. dadantii* (Antúnez-Lamas et al., 2009). The impaired swimming in the *rsmA* mutants demonstrated in this study may be caused by problems in bacterial chemotaxis-associated signaling.

The transcriptomic data revealed an up-regulation of genes involved in the tricarboxylic acid cycle, glycogen synthesis, glycogen metabolism, and citrate uptake in the

rsmA$^-$ and Δ*rsmA* mutants (II, III). However, the glycogen content in the bacterial cells, as visualized by an iodine-vapor staining assay, was observed to be lower in the *rsmA*$^-$ mutant (II). The results indicating genes involved in metabolism suggest that enhanced energy flux is occurring in the mutants.

When compared with studies of RsmA homologs in related enterobacterial pathogens, the findings of this study highlight the contrasting roles that RsmA homologs play in different bacteria. This study suggests that RsmA suppresses virulence-associated functions and the transition to a functional virulent state.

4.2.2 Comparing the RsmA regulon of *P. wasabiae* to those in related species

In this study, *P. wasabiae rsmA* mutants lost their swimming motility, despite the highly up-regulated genes involved in flagellar biosynthesis and hyperflagellation of the cells (II, III). Similarly, *rsmA* mutants of *Legionella pneumophila* and *X. campestris* display overproduction of flagella, and in the case of *X. campestris*, reduced motility as well (Chao et al., 2008; Molofsky and Swanson, 2003). Interestingly, in the case of *Salmonella enterica* sv. Typhimurium and *E. coli*, the abolished motility of *rsmA* mutants is a result of a complete lack of flagella (Lawhon et al., 2003; Wei et al., 2001). The *P. aeruginosa* PAO1 *rsmA* mutant also displays repressed expression of genes involved in flagellar biosynthesis and no motility on plate assays (Burrowes et al., 2006). In contrast, a *B. subtilis rsmA*$^+$ overexpressor has been reported to exhibit significantly less motility than the wild-type on plate assays (Yakhnin et al., 2007).

The up-regulation of glycogen biosynthesis, along with the increased glycogen exhaustion detected in the *P. wasabiae rsmA*$^-$ mutant, can be seen as contrasting behavior to what has been observed for *rsmA* mutants of *E. coli*. In *E. coli,* only an accumulation of glycogen has been detected (Timmermans and Van Melderen, 2009). These results, combined with up-regulation of genes involved in the tricarboxylic acid cycle and citrate uptake, indicate that the *rsmA*$^-$ mutant quickly utilizes its energy storage. Further metabolic analysis revealed that the *rsmA*$^-$ mutant also produces more 2,3-butanediol, a product of fermentation, which may also partly explain the lower pH detected in potato tuber tissue macerated by the *rsmA*$^-$ mutant compared with wild-type.

The *P. wasabiae rsmA* (deletion and knock-out) mutants exhibited a significantly delayed exponential growth phase and a reduced growth maximum (II, III). Slow and delayed growth was also detected in *rsmA* mutants in the animal pathogenic species *S. enterica* and *L. pneumophila* (Altier et al., 2000; Forsbach-Birk et al., 2004). In contrast, overexpression of *rsmA* homologs in *E. coli* and *P. mirabilis* is associated with inhibited growth (Liaw et al., 2003). Among other interesting aspects that were detected was the elongated form of *rsmA* mutants (II). This phenotype is in contrast to the elongation of *L. pneumophila* cells overexpressing RsmA (Molofsky and Swanson, 2003). In conclusion, a mutation of *rsmA* enhances the expression of virulence-associated genes in *P. wasabiae*, resulting in enhanced production of PCWDEs and plant maceration capabilities (II, III). Despite the enhancement of virulence-associated traits, the reduced growth, low energy state and impaired morphology

of the *rsmA* mutant causes reduced fitness, reduced microbial competition capabilities and impaired cellular multiplication *in planta* (II, III).

4.3 Analysis of the ExpA regulon of *P. wasabiae* (III)

4.3.1 Identification of extensive overlaps between the ExpA and RsmA regulons

The product encoded by *expA* is a useful research object because its importance in virulence seems particularly highly conserved among γ-proteobacteria. Other aspects of the *exp-rsm* system in the regulation of virulence, such as *expS* homologs, are not always apparent in related phytopathogens, for example, in *X. fastidiosa* (Shi et al., 2009).

Based on previous investigations regarding virulence regulation by RsmA and ExpA in *Pectobacterium* species, the transcriptomic data in this study revealed a high degree of overlap between the ExpA and RsmA regulons. Additionally, the data highlighted the opposing effects of *rsmA* and *expA* mutations on genes involved in virulence-associated functions, such as flagella biosynthesis, PCWDE production, T2SS and T6SS (Chatterjee et al., 2002, 1995; Cubitt et al., 2013; Cui et al., 2001; Hyytiäinen et al., 2001; Mukherjee et al., 1996). In addition to the importance of a functional ExpA for virulence, this study provides novel evidence for the importance of ExpA in swimming motility. The *expA* mutants, similarly to the *rsmA* mutants, were much less mobile on M9 minimal medium swimming motility plates. However, the swimming impairment in the *rsmA* mutants was more pronounced than in the *expA* mutants. As *rsmA* mutants are hyperflagellated and *rsmA* overexpression mutants have been shown to lack flagella, the results suggest that swimming motility is a function of more than simply the amount of flagella on the cell (II). Although *expA* mutants are unable to commence maceration of plant tissue in *in vitro* tobacco seedlings, they can persist as viable cells for days after inoculation onto the plant.

During late-logarithmic growth, approximately 40% of the *expA* regulon was oppositely affected in expression in comparison to the *rsmA* regulon. The microarray data further suggested that ExpA has the greatest effect on the virulence-associated transcriptome in the stationary growth phase, when genes encoding PCWDE, flagella and secretion systems exhibited reduced expression compared with wild-type, than in the logarithmic growth phase. However, this study mainly considers the transcriptomic data from the logarithmic growth phase due to its relatively strong statistical robustness.

Of further interest was the apparent regulation of one of the two T6SS, T6SS-1 (W5S_0962-0978), by ExpA via RsmA. However, there was no significant regulation of T6SS-2 (W5S_2418-2441) in any of the microarray data. T6SS-1 was up-regulated in the *rsmA* mutant and DM but down-regulated in the *expA* mutant. This observation may be partially explained by the evidence of relatively recent horizontal gene transfer (HGT) of T6SS-2, whereas T6SS-1 appears to have been part of the evolutionary history of *P. wasabiae* for a longer time (II, Figures 7 and 8). Although infection assay data suggested that the T6SS clusters play complementary roles in the maceration capabilities of *P. wasabiae*, the two T6SS may be involved in other functions as well. In addition to virulence, bacterial T6SS

have been implicated in competitive microbial interactions (Alteri et al., 2013; Blondel et al., 2013; Decoin et al., 2014; Haapalainen et al., 2012; Hachani et al., 2013). Initial bacterial T6SS-based competition assays against a variety of γ-proteobacteria yielded no significant difference between wild-type and a *P. wasabiae* mutant of both T6SS (data not shown). Similar to T6SS-1, relative up-regulation of the T2SS of *P. wasabiae* was detected in the *rsmA* mutant and DM, and down-regulation of the same operon was detected in the *expA* mutant. Interestingly, even though there was a clear impact on T2SS, T6SS-1 and several T1SS-associated genes, no significant change in expression of the T4SS of *P. wasabiae* was identified in any of the mutants tested. The T4SS may be regulated by other systems, and its importance for virulence remains unknown.

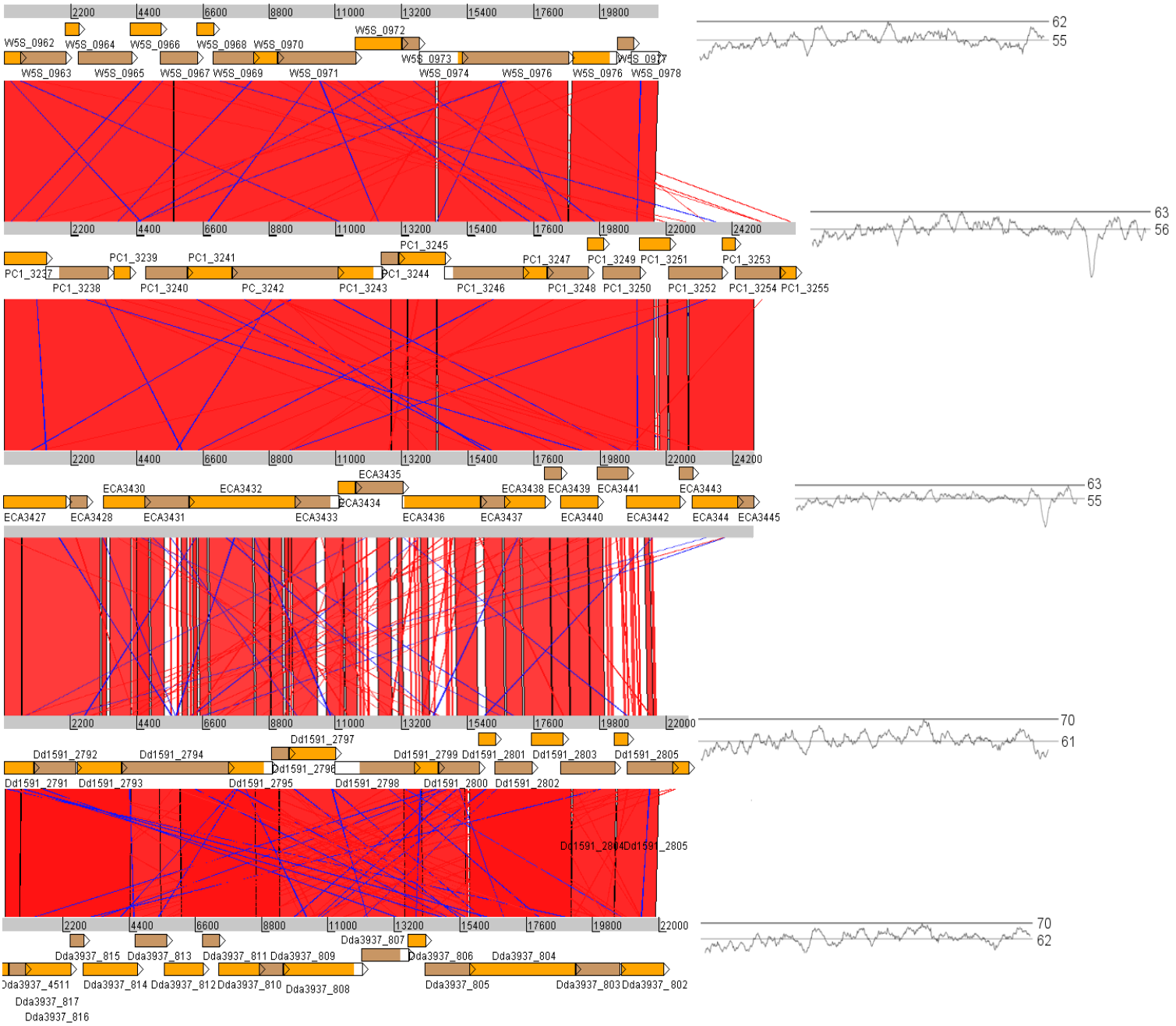
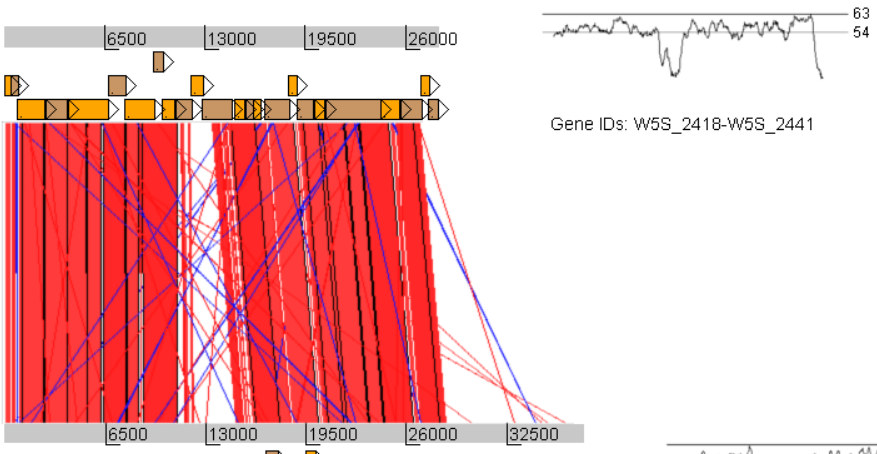


Figure 7. Comparison of the DNA sequence of the T6SS-1 operon of *P. wasabiae* SCC3193 (W5S_0962-W5S_0978) with the homologous region of *P. carotovorum* PC1 (now known as *Pectobacterium aroidea*, PC1_3237-PC1_3255), *Pectobacterium atrosepticum* SCRI1043 (ECA3427-ECA3445), *Dickeya chrysanthemi* Ech1591 (Dd1591_2791-Dd1591_2806) and *Dickeya dadantii* 3937 (Dda_4511, Dda_817-Dda_802) mentioned in the same order as from top to bottom in the picture. Red color indicates at least 88% matching sequences, and blue color indicates at least 88% reverse complement match. Graphs and numbers to the right indicate %GC across the T6SS operons. Orange and beige arrows indicate genes and the gene IDs are displayed above, below or to the sides.


6500
13000
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63
54
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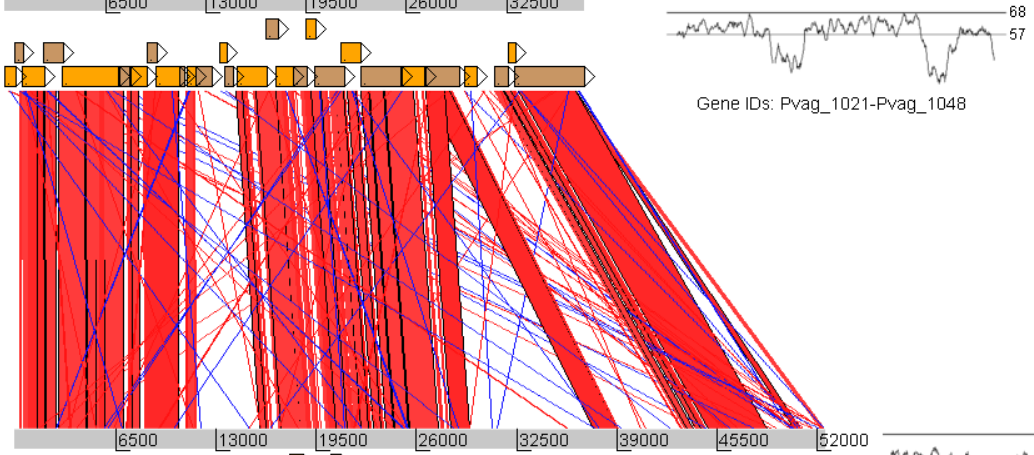
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19500
26000
32500
68
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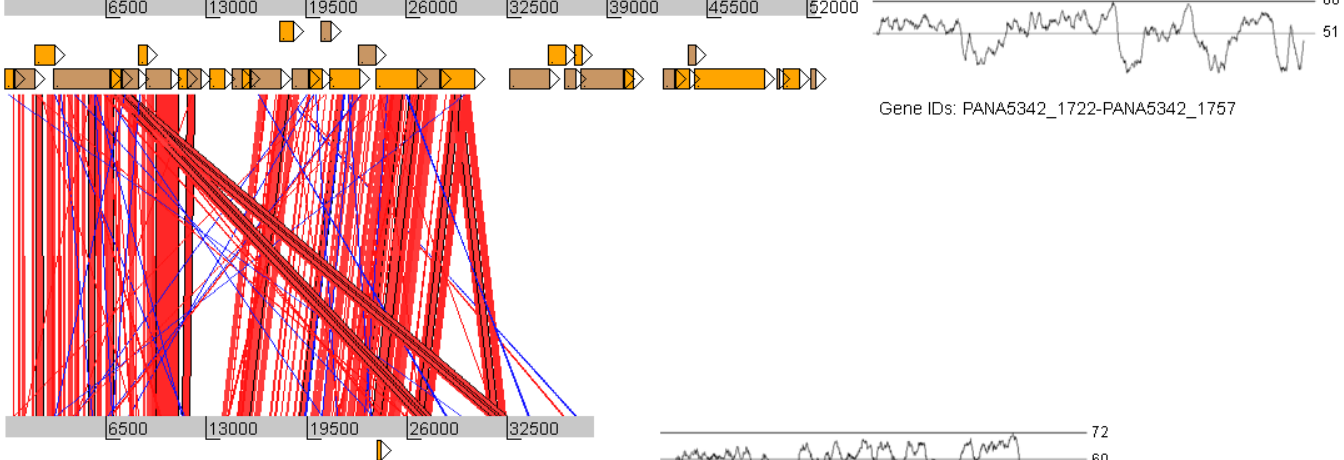
Gene IDs: Pvag_1021-Pvag_1048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45500
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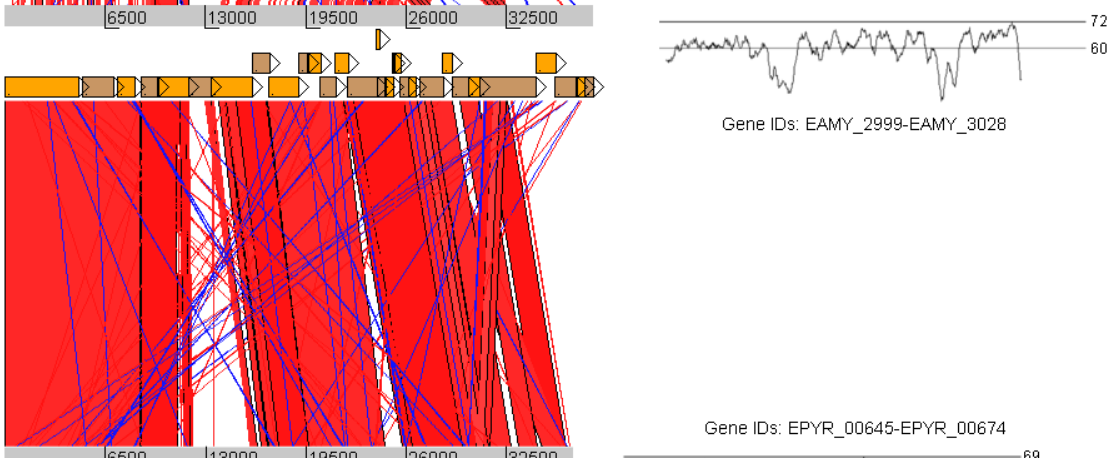
Gene IDs: PANA5342_1722-PANA5342_1757


6500
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19500
26000
32500
72
60
Gene IDs: EAMY_2999-EAMY_3028

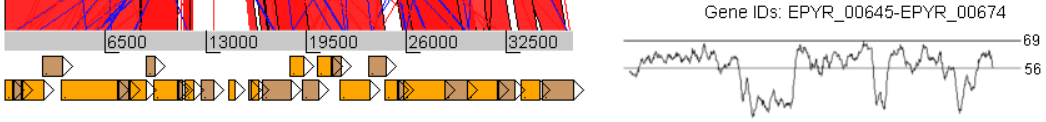
6500
13000
19500
26000
32500
Gene IDs: EPYR_00645-EPYR_00674
69
56

Figure 8. Comparison of the DNA sequences of the T6SS-2 of *P. wasabiae* SCC3193 (W5S_2418-W5S_2441) with *Pantoea vagans* (Pvag_1021-Pvag_1048), *Pantoea ananatis* (PANA5342_1722-PANA5342_1757), *Erwinia amylovora* (EAMY_2999-EAMY_3028) and *Erwinia pyrifoliae* (EPYR_00645-EPYR_00674) mentioned in the same order as from top to bottom in the picture. Red color indicates at least 88% matching sequences, and blue color indicates 88% reverse complement match. Graphs and numbers to the right indicate %GC across the T6SS operons.

Additionally, the operon encoding phosphonate uptake and metabolism (*phnGHIJKLMNP,* W5S_0593-W5S_601) was highly induced in the *rsmA* mutants and DM and correspondingly down-regulated in the *expA* mutant. *P. wasabiae* is a potato pathogen, and the active compound of the herbicide Roundup that is used on potato crops is a phosphonate known as glyphosate (Blackburn and Boutin, 2003). Thus, it was of interest to determine whether this compound affected the growth of a *P. wasabiae phn* mutant. However, no significant difference in the ability to survive in media containing the phosphonate compounds *N*-(phosphonomethyl)glycin (glyphosate) or phosphomycin (a fungal antibiotic) was detected between the *phn* mutant and wild-type. Furthermore, the *phn* mutant displayed no significant difference in virulence capabilities compared with wild-type in potato tubers.

The results from these investigations confirm that ExpA and RsmA have significant but opposing effects on an array of important virulence-associated functions.

4.3.2 Using the *expA-rsmA* double mutant as a tool for regulon analysis

The DM constructed for this investigation was based on the previously described *expA* mutant strain SCC3060 (Pirhonen et al., 1991). The *rsmA* gene in the DM was deleted and replaced with a chloramphenicol resistance cassette in the manner way as the *rsmA* single mutant was constructed. The mutant was subsequently used to help determine the relative hierarchy of ExpA and RsmA with regard to the genes regulated by them. In general, the DM was observed to be more similar to the *rsmA* mutant than the *expA* mutant in motility, PCWDE production and virulence. These results highlight the more central importance of RsmA for the bacterium. Regardless of the greater importance of RsmA, the DM was observed to be similar but not identical to the *rsmA* single mutant when comparing growth kinetics, Cel production and fitness during the infection of tobacco seedlings. The transcriptomic data revealed that approximately 56% of the genes detected as significant in the DM/wild-type comparison were similarly up/down-regulated in the *rsmA* mutant/wild-type comparison. In contrast, only 2.6% of the genes in the DM/wild-type data were oppositely regulated compared with the corresponding *rsmA*/wild-type data. Overall, 5.7% of the genes detected in the DM/wild-type comparison corresponded to similar regulation as the *expA* mutant/wild-type, whereas 13.3% displayed opposing up/down-regulation. In total, 1211 genes were observed to be significantly differentially expressed when comparing the wild-type to the DM at the late-logarithmic growth phase.

When comparing the gene expression data of all our microarray analyses of *rsmA* and *expA* mutants, as well as the DM, regions of the genome emerge where the effects of these regulators appear to be more pronounced (Figure 9), such as between the genes W5S_2100-W5S_2400. This region contains a variety of genes; for example 5 PCWDEs (W5S_2117, W5S_2136, W5S_2143, W5S_2146 and W5S_2358), the oligopeptide ATP-binding cassette (ABC) transporter encoding operon *oppABCDF* (W5S_2229-W5S_2234), the operon *rnfABCDGE* (W5S_2265-W5S_2270) encoding an electron transport protein, an RpoS response regulator (W5S_2224) and LysR regulator homolog (W5S_2356). Another common area of general up-regulation among the *rsmA* mutants is situated between genes W5S-2660-2700, containing genes encoding urea carboxylase, catalase-peroxidase and the antibiotic resistance protein MarR.

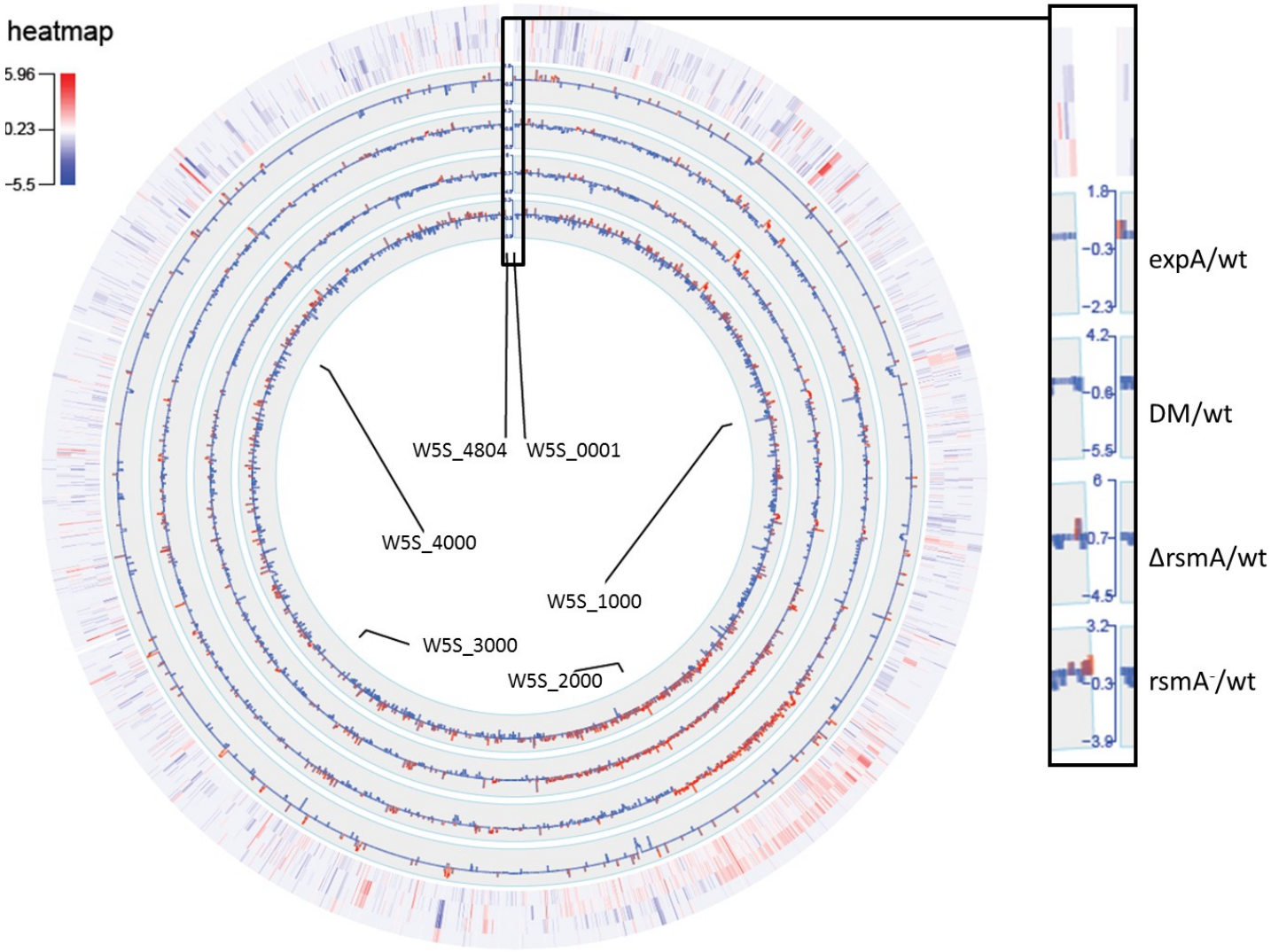

Figure 9. The combined microarray gene expression data of this study overlaid on the chromosome of *P. wasabiae.* The map order of gene expression comparisons is depicted to the right. Blue color signifies down-regulation while red color signifies up-regulation in the figure. The outer circle layer 1 symbolizes a heatmap in the same mutant/wild-type order as layer 2-5. In the figure, wt signifies wild-

type, and expA/wt depicts a significant gene expression ratio of the *expA* mutant to the wild-type, DM signifies the double-mutant, ΔrsmA signifies the *rsmA* mutant from article III and rsmA$^-$ signifies the *rsmA* mutant from article II. Numbers in the right map signify $\log_2$ fold change intervals of gene expression. The number 1 signifies the approximate position of gene W5S_0001, 2404 marks gene W5S_2404, and 4804 marks W5S_4804. The figure was made using the OmicCircos R-package (Hu et al., 2014).

In conclusion, the DM data suggest that ExpA exerts much of its regulation via RsmA and that it is hierarchically at a higher position compared with RsmA with regard to a majority of their corresponding regulons. The regulation of virulence by ExpA via RsmA is particularly apparent among many of the virulence-associated genes discussed in this dissertation (Figure 10). However, ExpA additionally seems to have an effect on virulence and cellular physiology that is independent of RsmA. The partial independence of ExpA from RsmA results in the double mutant being more virulent and exhibiting greater fitness *in planta* than either of the single mutants. However, the *P. wasabiae* mutants used in this thesis still fall short of the wild-type in terms of pathogenicity, highlighting the importance of a fully operational *exp-rsm* system for the virulence of *P. wasabiae*.

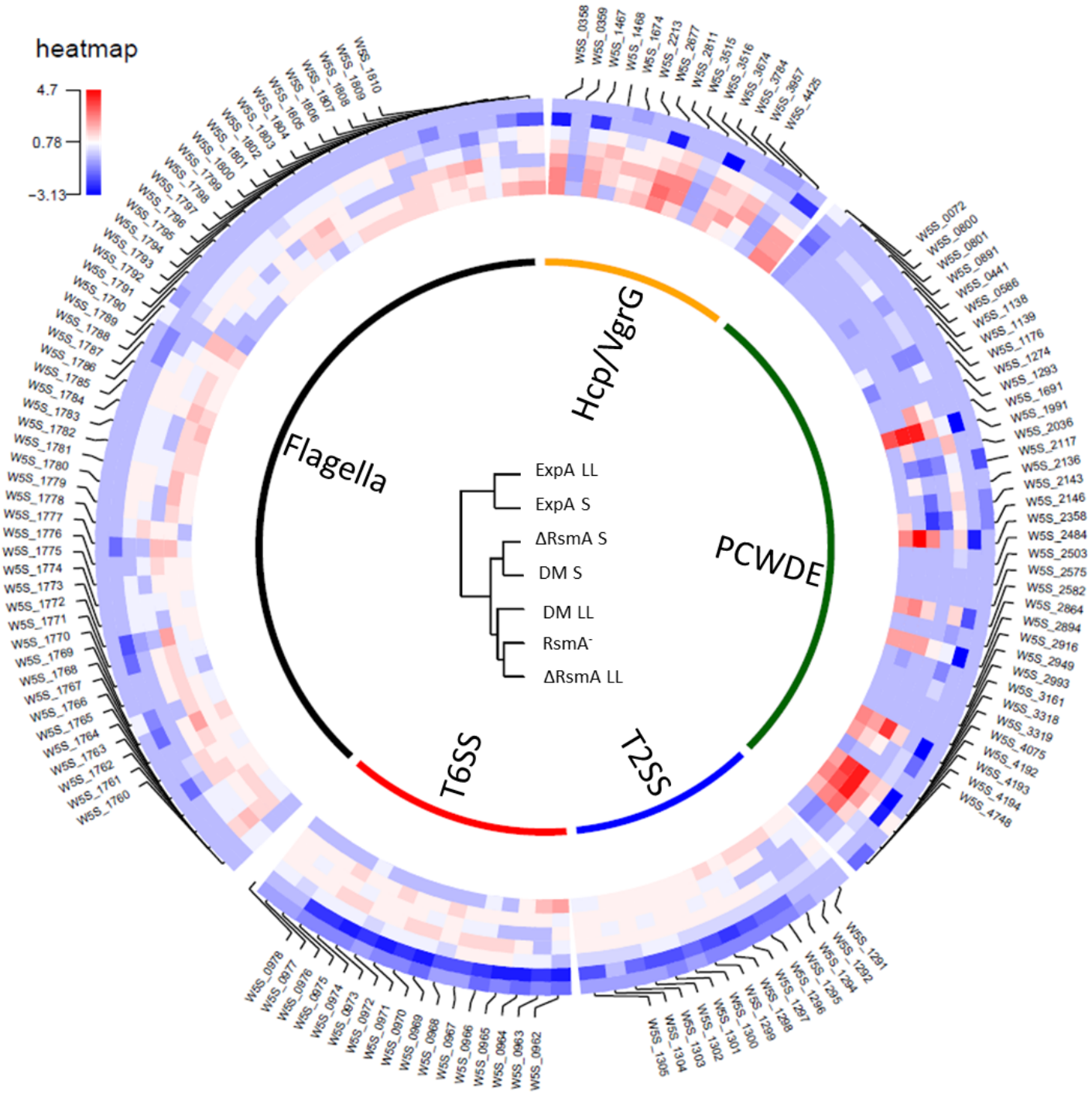

Figure 10. Clusters of virulence-associated genes in a circular heatmap based on the microarray gene expression data in this study. The color key of the heatmap denotes the $\log_2$ fold change. The cluster tree in the middle of the circle displays the order of mutant to wild-type comparison that the heatmap is ordered after (for example, the two outer rows of the map correspond to ExpA/wt data at different growth phases). ExpA signifies the data of the *expA* mutant. ΔRsmA corresponds to the *rsmA* mutant used in article III, whereas RsmA$^-$ corresponds to the *rsmA*$^-$ mutant used in article II. DM corresponds to the *expA rsmA* double mutant. S corresponds to the stationary growth phase, and LL to late logarithmic growth, where applicable. The figure was made using the OmicCircos R-package.

4.3.3 ExpA regulates genes independently of RsmA (III)

By using the DM in the transcriptomic experiments, work could begin to selectively identify genes that may be regulated directly or indirectly by ExpA independently of RsmA. In total, at the logarithmic growth phase, 37 such genes were detected. The identified genes seemed to be involved in a wide variety of cellular functions, such as oligogalacturonide metabolism, replication and electron transport. Of particular interest was *kdgR*, which encodes an important virulence regulator in *Pectobacterium* species (Hyytiäinen et al., 2001; Liu et al., 1999). The transcriptomic data reported that *kdgR* was down-regulated in the *expA* mutant and DM compared with wild-type, but not in the Δ*rsmA* mutant. Upon further analysis, RT-qPCR revealed up-regulation of *kdgR* in the Δ*rsmA* mutant, whereas confirming down-regulation in *expA* and the DM. In the related soft rot pathogen *Dickeya dadantii*, *kdgR* is a negative regulator of the *togMNAB* operon, which is associated with the uptake and metabolism of oligogalacturonides (Hugouvieux-Cotte-Pattat et al., 2001). Despite the down-regulation of *kdgR*, simultaneous down-regulation of the *togMNAB* operon in the *expA* mutants and DM was observed. However, the *expA* mutant exhibited similar growth kinetics as that of the wild-type, regardless of the down-regulated synthesis of the oligogalacturonide uptake machinery in a medium where oligogalacturonide was the carbon source.

The operons encoding the cytochrome o ubiquinol oxidase complex (W5S_3266-3270) and the pyruvate dehydrogenase complex (W5S_3892-3895) were up-regulated in the *P. wasabiae expA* mutant. These two operons have been associated with the redox state of bacterial cells and with virulence in the animal pathogen *Streptococcus pneumoniae* (Alverdy et al., 2000; Beier and Gross, 2006; Ogasawara et al., 2007; Spellerberg et al., 1996). Nonetheless, the potential effects on the virulence of *P. wasabiae* exerted by these operons and the cell redox state are unclear and remain to be elucidated.

In conclusion, this study presents an in-depth characterization of ExpA and RsmA in *P. wasabiae*. ExpA regulates many virulence functions via RsmA but seemingly has a primary effect on metabolism and cellular multiplication independently of RsmA. This effect might be the cause of the increased fitness of the DM *in planta*, likely due to its greater maximum growth and earlier exponential phase.

4.4 Biological activity of short oligogalacturonides (IV)

4.4.1 Effects of short oligogalacturonides on plant defense and growth

As plant cell walls are degraded by the PCWDEs secreted by bacteria, such as *P. wasabiae*, fragments of digested cell wall components are released into the plant apoplast. Some of these released components originate from proteins or polysaccharides and are commonly referred to as damage-associated molecular patterns (DAMPs). When sensed by nearby cells, DAMPs induce responses, such as those related to plant defense (Bellincampi et al., 2000; Galletti et al., 2011). Previous whole transcriptome analyses of plants treated with oligogalacturonides (OGs) have mainly studied OGs with a degree of polymerization (DP)

>10 (long OGs). So far, long OGs have been regarded as the most effective in inducing defense signaling in *A. thaliana* (Denoux et al., 2008; Ferrari et al., 2007; Moscatiello et al., 2006). However, previous data have also indicated a role for short OGs (OGs with a DP <10) in the plant defense response in potato, tobacco and tomato (Ferrari et al., 2007; Norman et al., 1999; Simpson et al., 1998; Weber et al., 1996). Thus, in this study plants were treated with 1/2 Murashige and Skoog (MS) medium supplemented with commercially available trimeric OGs (trimers), a mixture of 1/2 MS supplemented with a mixture of OGs with a DP between 2-9 (short OG-mix) and a mock treatment (only 1/2 MS). Three hours after the treatment, RNA was extracted from the treated plants and subsequently sequenced. The RNA sequencing data demonstrated that the treatment of plants with short OGs (DP<10) had an impact on a wide array of defense-related genes, as well as on genes involved in general plant metabolism. At 3 h post-treatment, there was a significant change in the expression of 700 genes in response to trimer treatment in comparison to the mock treatment, whereas treatment with the short OG-mix induced a relative change in the expression of 3891 genes. This study suggests that there are differences in the plant response to mixtures of OGs with varying DP or to OGs of a single DP.

A gene set enrichment analysis (GSEA), based on genesets provided by the Plant Geneset Enrichment Analysis Toolkit, was performed on the RNA sequencing data (Yi et al., 2013). The genesets derived from the Gene Ontology Consortium database (GO) were utilized first-hand for the comparative analysis of transcriptome data in this work (Ashburner et al., 2000). The GSEA results revealed a general trend of down-regulation of genesets associated with metabolism and plant development and up-regulation of genesets associated with the pathogen defense response, phytohormone signaling and stress. For example, short OGs induced jasmonic acid biosynthesis (GO:0009695), ethylene response (GO:0009723), oxylipin biosynthesis (GO:0031408), response to wounding (GO:0009611) and immune effector processes (GO:0002252), but impaired cellular component biogenesis (for example, GO:004225 - ribosome biogenesis and GO:0070271 - protein complex biogenesis), organelle organization (for example, GO:0009657 - plastid organization, GO:0006996 - organelle organization, GO:0009668 - plastid membrane organization and GO:0009658 - chloroplast organization), energy metabolism (for example, GO:0015979 - photosynthesis, GO:0005996 - monosaccharide metabolic process and GO:0019684 - photosynthesis light reaction) and development (for example, GO:0009790 - embryo development, GO:0048316 - seed development and GO:0044085 - cellular component biogenesis). The clear division of geneset regulation demonstrated by the GSEA was particularly apparent in the trimer-treated plants, whereas plants treated with the short OG-mix exhibited a larger and more diverse collection of affected genesets. Furthermore, the short OG-mix GSEA results demonstrated an overall greater overlap between functionally similar up- and down-regulated genesets than the trimer data. The short OG-mix treatment caused the up-regulation of genesets involved in SA, JA, ET and ABA production and signaling, along with the down-regulation of genesets involved in auxin transport. The trimers significantly affected genesets associated with JA and ET. The differences in GSEA output between the two short OG treatments may be partially explained because of the varied length of the OGs in the short OG-mix (DP of 2-9) compared with the trimers (DP=3). OGs of different length may potentially bind different receptors and activate different biological pathways.

An RT-qPCR time series analysis was performed to confirm the RNA sequencing results of this study. In addition, the aim of the RT-qPCR was to investigate interesting marker genes involved in phytohormonal signaling and to compare the effects of short OGs on the expression of genes studied in long OG-based investigations. Plants were treated similarly as for the RNA sequencing, and samples were taken at 1, 3, 7, 12 and 24 h post treatment. The RT-qPCR data revealed a general up-regulation of the wounding-associated genes *PER4* and *CYP81F*, along with the OG receptor *WAK1* at 3 h and later time points. The gene *PAD3*, previously reported as essential for the OG response in plants, was also up-regulated at 3 h and onwards (Ferrari et al., 2007). The JA response-associated gene *WRKY40* was up-regulated, whereas the ABA-associated marker *RAB18* was down-regulated. *PGIP1*, which is involved in the response to fungal polygalacturonases, was down-regulated at 1 h but up-regulated at 3 h and 12 h. *CML41*, which is putatively involved in plant defense, was also up-regulated by the short OGs. Overall, the gene expression RT-qPCR data were similar between the short OG-mix and the trimers. However, the expression fold change of the genes of interest was in general more pronounced for the short OG-mix. In addition, the RT-qPCR gene expression data for short OGs was generally similar to that of long OGs, mainly at the 3 h time point and later.

Assays for plant pathogen resistance and growth retardation were used to test the induced immune response and impaired plant development indicated by the gene expression data. *In vitro* plants pre-treated with trimers for one day before flood inoculation with *P. carotovorum* ssp. *carotovorum* SCC1 were slightly more resistant to infection possibly due to the activation of DAMP signaling and SAR. The elicitation of defense by trimers in the *in vitro* plants resulted in lower bacterial propagation *in planta* compared with wild-type. Furthermore, plants treated *in vitro* with trimers or the short OG-mix also exhibited significant growth retardation in comparison to plants treated with a mock suspension. However, the growth retardation was more significant in plants treated with the short OG-mix. Increasing the concentration of trimers to more than 200 µM caused the plants to die instead of exhibiting reduced retardation at the same level as those treated with the short OG-mix. The results of the phenotype assays highlight the differences in gene expression. The trimers seem to clearly down-regulate genes involved in developmental pathways and induce those involved in defense. In contrast, the short OG-mix produces a more varied response including inhibition of plant growth and unclear defense induction. The results of the physiological assays and RNA sequencing further suggest that mixtures of OG with different DPs trigger plant responses distinctly compared with OGs of a single type of OG.

4.4.2 Comparison of the transcriptomes of plants treated with short or long oligogalacturonides

One of the aims of this work was to further expand the knowledge of the previously identified effects of OGs and DAMP-triggered signaling in *A. thaliana*. Comparative transcriptomics of *A. thaliana* treated with long OGs (DP>10) have been performed in past studies by the use of gene expression microarrays (Denoux et al., 2008; Ferrari et al., 2007; Moscatiello et al., 2006). The data from these studies were subsequently combined with the RNA sequencing

data generated in this study for a meta-analysis of the transcriptomic effects of OGs in *A. thaliana*. Of particular interest were the microarray-based gene expression studies of Ferrari *et al*. (2007) and Denoux *et al.* (2008), which were performed on plants that were treated similarly as those used for the RNA sequencing experiment of this study. Comparisons between the data of these two investigations (using a P-value of <0.01 as a cut-off for significance) and this study revealed 126 differently expressed genes affected by short and long OGs. Additionally, at 3 h, 335 genes were differently expressed only in plants treated with either trimers or the short OG-mix, after subtracting genes with significantly altered expression after treatment with long OGs. Adding the comparative gene expression microarray data from the OG treatment of *A. thaliana* hypocotyl cells in a suspension culture by Moscatiello *et al.* (2006), a total of 10 genes were affected across all studies (Figures 11 and 12). The 10 genes were identified as up-regulated in this work and in the work of Denoux *et al.* (2008) and Ferrari *et al.* (2008) but down-regulated in the study of Moscatiello *et al.* (2006). Notably, the gene expression study by Moscatiello *et al.* (2006) was performed in very different conditions to those used in this work, which highlights how the experimental set-up can influence the results.

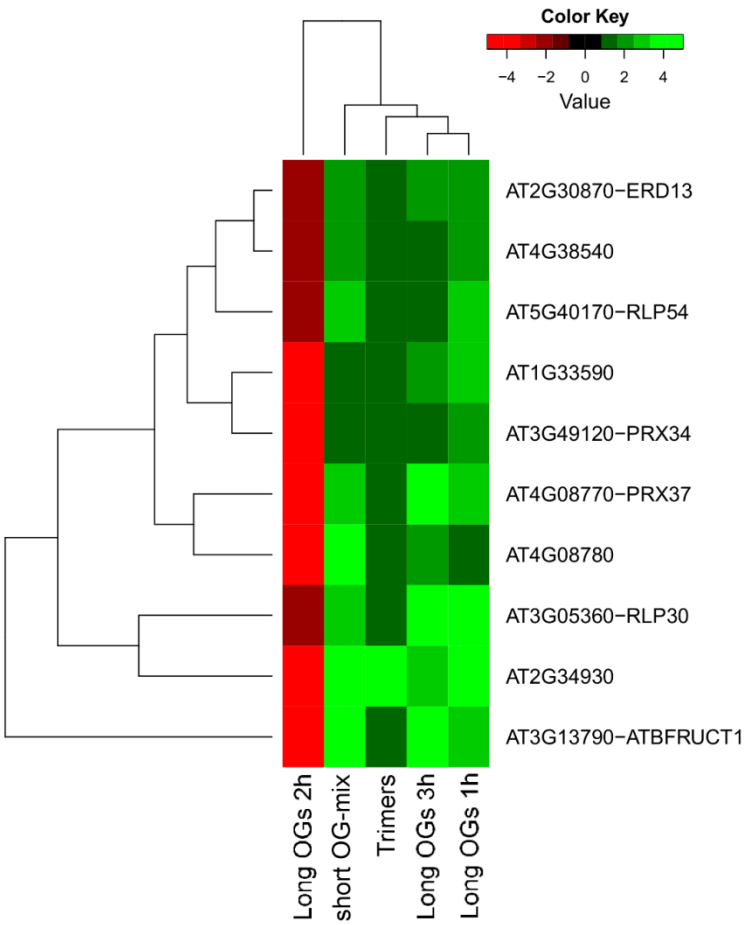

Figure 11. Heatmap overview of the 10 genes identified in this study using plants treated with trimers (Trimers) and short OG-mix (short OG-mix), the microarray study of long OG treatments by Denoux *et al.* (2008) at 1 h (Long OGs 1h) and 3 h (Long OGs 3h), and the study by Moscatiello *et al.* (2006, Long OGs 2h). The color key depicts the color code for fold change.

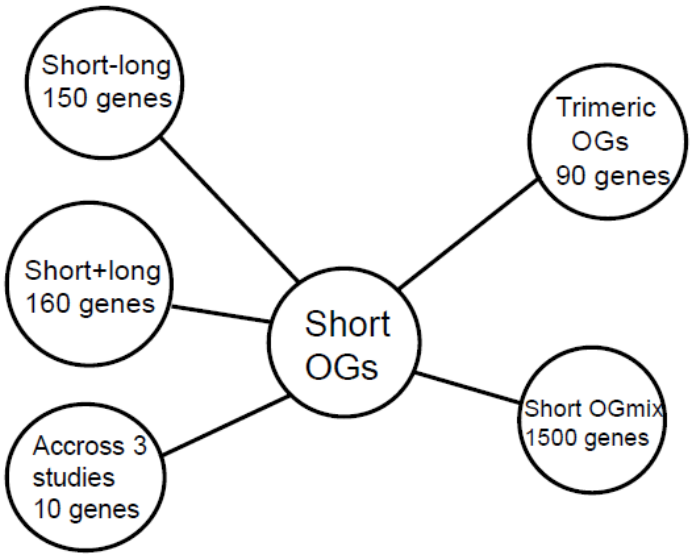

Figure 12. Overview map of comparisons made between transcriptomic data of the analysis described in this dissertation and previous microarray experiments of plants treated with OGs with a DP>10 (long OGs). The circle marked Short OGmix displays the number of genes with significantly different expression when comparing plants treated with the OG-mix to mock-treated plants. Similarly, the circle labeled Trimeric OGs contains the number of genes identified when comparing trimer treatment to mock treatment. Short-Long displays the genes identified as putatively affected by only short OGs. Short+long signifies genes affected by short and long OGs.

GSEA was also performed on the various combinations of the gene expression data of this study and the data from Ferrari *et al.* (2007) and Denoux *et al.* (2008). The overlapping genes with significantly altered expression by both long (at 1 h and 3 h) and short OGs (at 3 h) yielded a GSEA result of 67 up-regulated, and 70 down-regulated genesets ($P<0.05$). The GO-based GSEA data of long and short OGs indicated, in similar fashion as the GSEA results based on only the short OG data, that genesets involved in development, photosynthesis and energy metabolism were down-regulated by both long and short OG treatments. Conversely, the GSEA demonstrated that genesets involved in JA and ET-signaling and responses to stress and immunity were up-regulated (Figure 13). However, differences between long and short OGs were also apparent. For example, the GSEA output of the long OGs based on the study by Ferrari *et al.* (2007) and Denoux *et al.* (2008) at 3 hours (using FDR<0.01 as cutoff) revealed an up-regulation of genesets such as systemic acquired resistance (GO:0009627), response to bacterium (GO:0009617) and response to biotic stimulus (GO:0009607), while down-regulation of genesets such as epidermis development (GO:0008544), organ development (GO:0048513) and system development (GO:0048731). These genesets were not detected as significantly affected in the RNA sequencing data of this study. Additionally, genesets involved in JA-signaling (GO:0009694 and GO:0009695) and oxylipin biosynthesis (GO:0031408) were found to be induced by

short OGs. These differences may further indicate that treatment with long OGs or short OGs trigger different responses.

The RT-qPCR results of this study corresponded to the data of the same genes from long OG treatments (Denoux et al., 2008). For example, the genes *WAK1*, *WRKY40*, *PER4*, *PGIP1, CML41* and *PAD3* were up-regulated by both long and short OGs at time-points of 3 h and later. However, *WRKY40*, *PER4*, *PAD3* and *PGIP1* were down-regulated at 1 h in plants treated with short OGs, opposite to what was found for long OGs. Thus, the treatment of *A. thaliana* seedlings with short OGs has a generally similar effect as the treatment with long OGs, for marker genes associated with defense and phytohormonal signaling. However, at 1 h the fold changes in expression differed significantly for several genes when comparing data from plants treated with long OGs to those treated with short OGs. The impact of long OGs was more pronounced on overall gene expression, particularly in comparison to trimers.

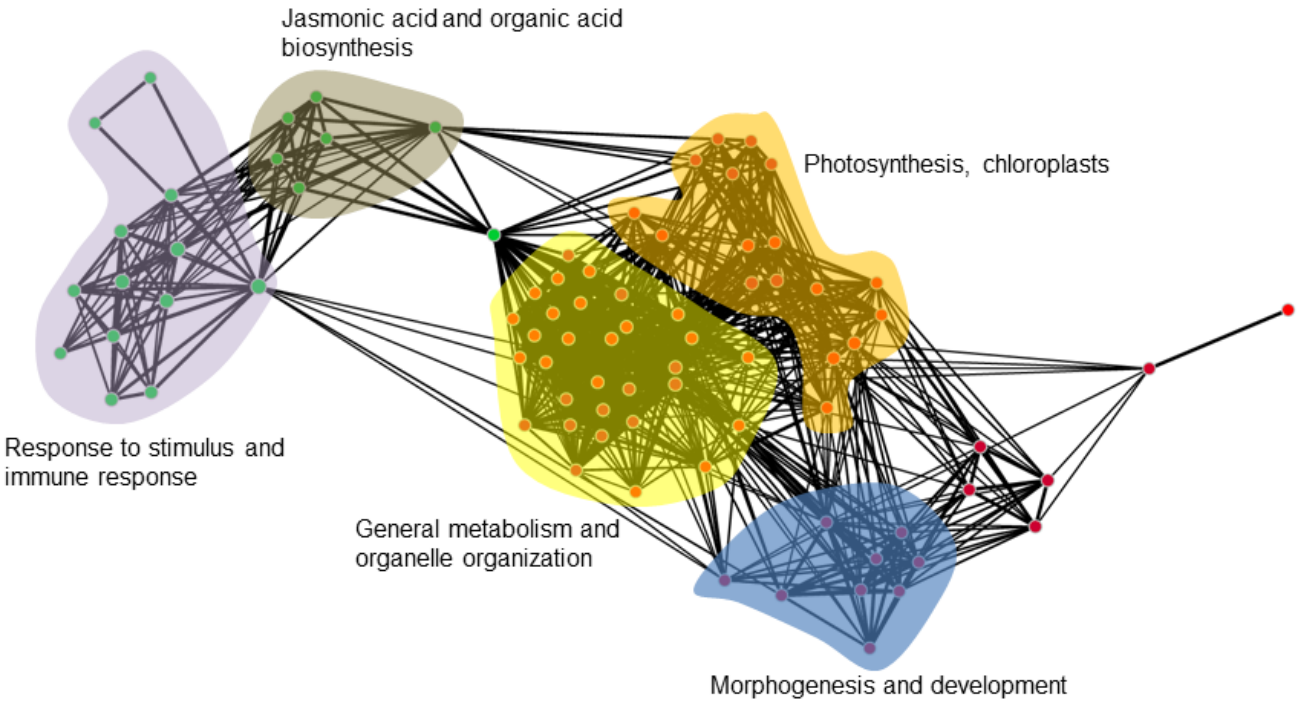

Figure 13. GSEA result (GO-related genesets, P<0.05) of the gene expression data from RNA sequencing of plants treated with trimers and short OG-mix, combined with microarray data of plants treated with long OGs at 1 h and 3 h as reported by Denoux *et al.* (2008). Colored areas indicate the manual labeling of the general processes the genesets are involved in. Green colored nodes indicate an up-regulated geneset, and red colored nodes indicate a down-regulated geneset.

Previous studies have suggested that OGs operate independently of the SA, JA and ET signaling pathways with regard to plant defense induction (Denoux et al., 2008; Ferrari et al., 2007). However, long OGs have also been indicated to induce genes associated with JA, ET and SA signaling while down-regulating ABA signaling at early time points (Denoux et al., 2008). Overall, the data in this study indicated a clear up-regulation of genes involved in

the signaling and synthesis of JA and ET by treatment with the short OG-mix and trimers. In addition, the short OG-mix induced the up-regulation of genesets associated with ABA and SA signaling. These results indicate that trimers and the short OG-mix induce JA and ET signaling pathways similarly. However, the induction of various genesets related to phytohormonal signaling was more pronounced and varied in the plants treated with the short OG-mix. Since ABA, ET, JA and SA have been reported to operate antagonistically (except for ET and JA which do not generally antagonize each other), and one may speculate that OGs in general have an impact on the cross talk between these phytohormones. As genes involved in ABA signaling are both up- and down-regulated by the short OG-mix treatment, the phytohormone signaling response to the short GO-mix seems complicated. The induction of JA by wounding and the associated DAMP-induced activation of plant defenses has previously been established for *A. thaliana* (Titarenko et al., 1997; Wang et al., 2000).

Because genes and genesets associated with ROS production were induced by short OGs, whether short OGs elicit an accumulation of ROS *in planta* was tested. Thus, a luminol-based assay was used to measure the ROS burst in plants treated with trimers, the short OG-mix or a mock suspension (Tateda et al., 2014). Early time point measurements of Arabidopsis leaf discs indicated a significant ROS burst in plants treated with the short OG-mix. However, this was not detected in plants treated with trimers. Additionally, the RNA sequencing data revealed that a marker gene for ROS burst, *AtRBOHD* (encoding a NADPH oxidase), was significantly up-regulated by short OG-mix treatment but not by trimers. The ROS burst measurements, combined with differences in gene expression data, suggest that the short OG-mix and trimers affect the plant immune response in different ways. The short OG-mix treatment overall affects gene expression on a larger scale than the trimer treatment, for example the regulation of genesets involved in ROS metabolism, plant development and growth. However, ROS burst mediated by *AtRBOHD* has been indicated to not be necessary for induction of OG-induced defense response against *Botrytis cinerea* (Galletti et al., 2008).

In conclusion, the transcriptomic data revealed that short OGs modulate a significant part of the plant transcriptome, and that they affect plant defense pathways and plant growth. The meta-data analysis further exhibited the overlaps and differences in modulation of defense and growth signaling between short (DP<10) and long OGs (DP>10). As the bacterial infection progresses the digestion of pectin derivatives continues, eventually resulting in the domination of short OGs. Thus, one might expect it to be practical for the plant to distinguish between longer and shorter OGs, as they may arise during different stages of bacterial infection. In general, it seems long OGs have a wider and more profound impact on the plant defense and growth response, based on a comparison of transcriptomic studies (Denoux et al., 2008; Ferrari et al., 2007). Additionally, the short OG-mix had a larger influence on the global transcriptome than the trimers. The majority of the trimer-triggered gene expression data overlapped with that triggered by the short OG-mix. Plant response to both types of short OGs share similarities in general up-regulation of plant defense-associated genes, and down-regulation of genes related to development and growth. However, the short OG-mix treatment overall affects gene expression in plants more than the trimer treatment, including the regulation of expression for genesets involved in ROS metabolism, plant development and growth. Short OGs should be regarded as a part of the signaling that occurs during the interactions between plants and necrotrophic pathogens. The results discussed here

suggest that treating plants with a mixture of OGs with varying DP is more potent in eliciting responses than treatments with OGs of only one type of DP. Furthermore, there may be different receptors for different lengths of OGs.

This work expands our current understanding of plant-pathogen interactions, combining novel results from each side. Necrotrophic soft rot bacteria react to their environment inside the plant, causing the activation of regulators that coordinate the induction and repression of virulence-associated genes. As the bacteria commence their infection in the plant using their repertoires of secretion systems and PCWDEs that degrade the plant cell walls, DAMPs are released and sensed by the plant. The sensing of DAMPs, such as short OGs, elicits defense signals in the plant, enhancing pathogen resistance and reducing growth and development (Figure 14).

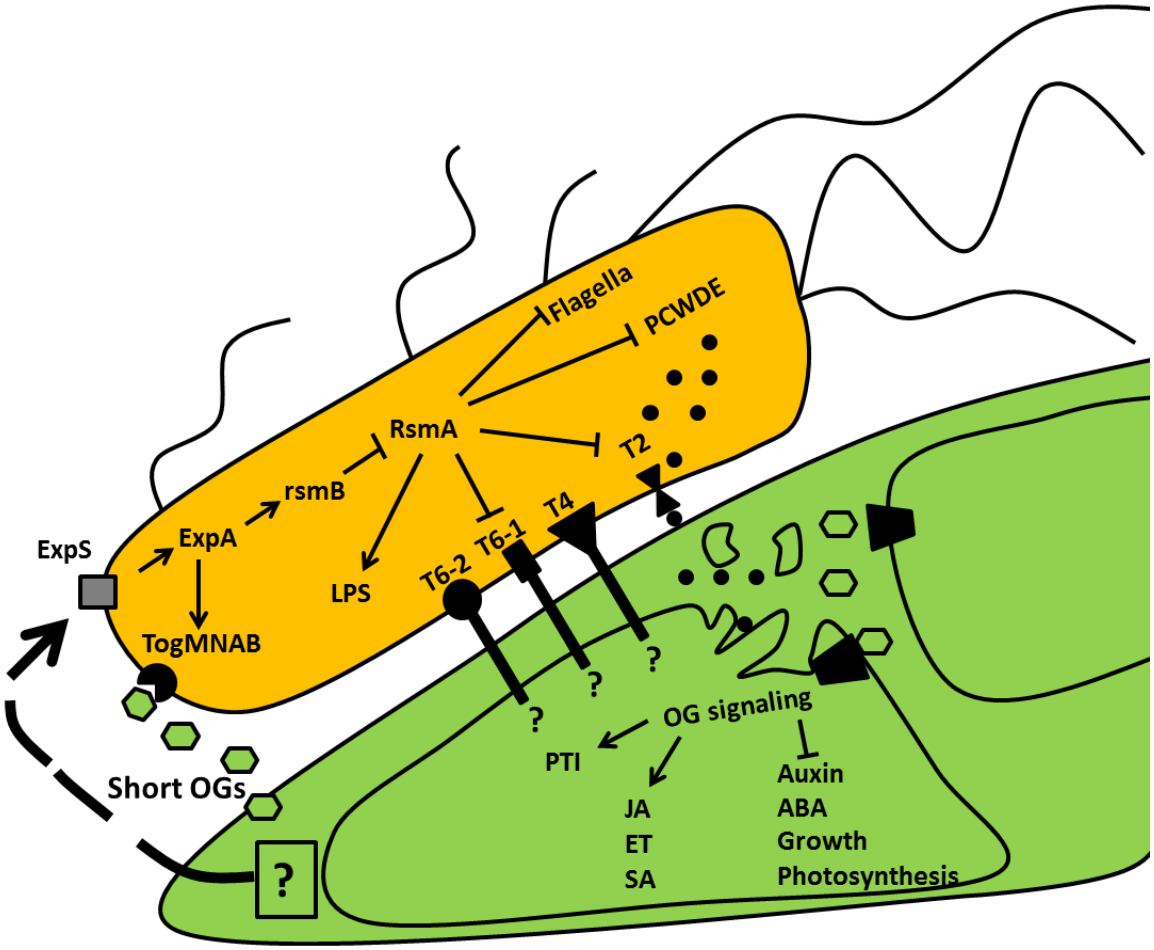

Figure 14. An overview of the putative interactions between *P. wasabiae* and *A. thaliana* during infection based on this work. In the figure, T6-2 refers to T6SS-2, T6-1 to T6SS-1, T4 to T4SS, T2 to T2SS, T1 to T1SS, and PCWDEs to plant cell wall degrading enzymes; ? signifies an unidentified but possibly plant-derived signal for activating ExpS; and OGs refers to oligogalacturonides. Arrows indicate induction, and truncated lines indicate repression.

5 CONCLUSIONS AND FUTURE PROSPECTS

The work in this dissertation has strived to highlight different aspects of what may occur in the host and pathogen during plant infection by soft rot bacteria. To achieve this goal, transcriptomic analyses using different but scientifically valid methods were combined with physiological assays. In addition, functional genetics and genomics were used for analyzing the soft rot model in this work. In articles I, II and III, this study characterizes aspects of the bacterial regulation networks that are important for virulence. The two central regulators, ExpA and RsmA, have been revealed to modulate the expression of important virulence-associated genes encoding, for example, T2SS, T6SS, PCWDEs, flagella and chemotaxis-related proteins in *Pectobacterium wasabiae*. In addition to the traits directly associated with virulence, ExpA and RsmA also regulate aspects of general metabolism and the cell cycle, further modulating bacterial fitness. The output of the microarray analysis has provided novel information on the *exp-rsm* regulatory network in *Pectobacterium* ssp. Due to the many studies on the subject and the continuous increase in data, further integration and comparison of the data between the related pathogens may be needed.

The functional assays of motility suggest that it is a very finely balanced aspect of the bacterial lifestyle, as both the *P. wasabiae rsmA*$^{-}$ (knockout), Δ*rsmA* (deletion), and *expA* mutants display abolished swimming motility. However, the swimming motility of the *rsmA* mutants was more severely reduced compared with the *expA* mutant. An investigation into the organization and communication of bacterial swimming may be of importance in understanding how the bacteria coordinate high-density populations.

The results of this dissertation serve to further establish the roles and hierarchies of ExpA and RsmA in regulation, where ExpA affects the transcription of many virulence-associated genes due to its regulation of RsmA. By constructing an *expA rsmA* double mutant, a novel type of mutant for *exp-rsm* research, the fact that ExpA also regulates part of bacterial metabolism (for example, part of the electron transport chain) independently of RsmA was revealed. Thus, the mutation of *expA* serves to impact the growth kinetics in the double mutant, which in this case enhances the fitness of the double mutant during infections. However, the exact mechanism used by ExpA to bind DNA, the protein structure and its binding motif remain to be fully understood. The regulons of proteins interacting with the *exp-rsm* network, for example, KdgR, RpoS, ExpI, ExpR, and RsmC, remain to be fully characterized and hierarchically positioned in the general systemic regulation of soft rot bacteria. Further transcriptomic analysis could help develop this regulatory map. As transcriptomic analysis becomes cheaper, more in-depth time scale studies could further identify when certain parts of the *exp-rsm* regulation turns on or off and the impact of QS over time on this system. Additionally, there may be other important small ncRNAs aside from the product of *rsmB* to be detected and characterized in *P. wasabiae*, possibly by RNA sequencing.

As the soft rot bacteria macerate the plant tissues during infection, the digestion of pectin releases OGs. These fragments are recognized by the plant and induce defense responses, and growth reduction of the plant. The results of the transcriptomic analyses and phenotypic assays of this study serve to establish that the modulation of pathogen resistance and plant development by short OGs is similar to that of long OGs. Short OGs can be used to

activate the systemic defense of plants. For example, this study shows that short OGs induce resistance to the necrotrophic pathogen *Pectobacterium carotovorum* ssp. *carotovorum* SCC1 and reduce plant growth. While the plant commences to trigger defense and phytohormonal signaling (for example, SA, JA and ET), the expression of genes associated with biosynthetic pathways and growth are reduced. The continued characterization of the overlaps and mechanics of OG signaling may prove to be a fruitful area of investigation in the future. To date, only one receptor for oligogalacturonides has been characterized. The data combined from this and other studies of the transcriptomics of OGs contain gene expression regulation of several relatively uncharacterized receptors and membrane-associated proteins. These proteins could be investigated further to unveil their potential involvement in OG perception, possibly by utilizing immunoprecipitation and radiolabeled OGs or by performing targeted mutagenesis for assaying insensitivity to OG treatments.

6 ACKNOWLEDGEMENTS

The research of this study was performed at the University of Helsinki, in part at the Division of Genetics, Department of Biosciences, and in part at the Department of Agricultural Sciences, under the supervision of Professor Tapio Palva, and University lecturer Minna Pirhonen. The work was funded supported by the Niemi Säätiö, Ellen, Hjalmar och Saga Waselius' Stipendiefond, the Academy of Finland (Center of Excellence program 2006-2011, grants 213509, 129628, 136470, 120821, and 128566), the Finnish Doctoral Program in Plant Sciences, Biocenter Finland, Biocentrum Helsinki, and by the University of Helsinki.

I wish to give my deepest thanks to my supervisors Professor Tapio Palva and University lecturer Minna Pirhonen for the opportunity to perform research within the exciting field of plant-pathogen interactions. Their guidance and support over the years has provided a solid foundation and inspiration for my future career.

I am grateful towards the Division of Genetics and the Department of Agricultural Sciences for the work-spaces provided and the excellent environment and atmosphere for scientific research.

I thank my thesis committee, Professor Benita Westerlund-Wikström and Professor Jari Valkonen (and the late Professor Kielo Haahtela) for their encouraging words and assistance reviewing my progress during the road to my dissertation.

I am very grateful to my dissertation pre-examiners Professor Harri Savilahti and Professor Kristina Lindström for taking the time to examine and evaluate this dissertation.

I am thankful for my co-authors and collaborators from the University of Tartu; Liis Andresen, Viia Kõiv and Andres Maë. From South Korea, I wish to acknowledge the fruitful collaboration with Gir-Won Lee, who provided me with guidance in R-programming, and Professor Yong-Hwan Lee. I am also indebted to Panu Somervuo for his aid in bioinformatics, R-programming and microarray analysis.

I give my gratitude to the research groups I have been part of, and for the good people I have had the privilege to spend time with during my time here. In particular; Pär Davidsson, who has been a good source of collaboration in science, and a friend to share activities with outside of the University. Mehment Ali Keceli, a colleague one who is a great source for activities such as movies, sports or games. Tarja Kariola, I wish to thank for much help, not only in research and scientific writing, but also for help in exploring interesting restaurants around Viikki. Hanne Mikkonen, who has remained a source of nice parties and general talk in the office. Mantas Survila, always a pleasure to have along for activities in and outside of the lab, and Sunday lunches too! Karen Sims-Huopaniemi for aid in many bureaucratic and practical questions surrounding PhD studies, and help with installation in Helsinki during my early time in Finland. Furthermore, I wish extend thanks to colleagues Maria Piisilä, Outi Niemi, and Ville Pennanen.

I also wish to thank my friends from outside my research group: Eric Pedersen, who has been a great partner for travelling around in Europe. I'm glad for the friendship of Filip Mundt and Tomas Persson, for many discussions about life and science, and refreshing point-of-views.

I give thanks to my parents Mats and Maria-Elena for their support and giving me energy to continue during this time. I am also grateful to my siblings Tomas, Sofia, Linus, Teresa and Henrik. I also thank Petter and Gloria. Thanks to my relatives, my cousin Johannes for providing rewarding discussions while being in similar situations. Home was never really far away, geographically or emotionally.

Thank you to Sirkku, for your support and closeness, you helped me to finalize this period of my life in more ways than you know!

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