VIRULENCE OF SOFT-ROT ENTEROBACTERIA AFFECTING POTATO

DOCTORAL THESIS IN PLANT PATHOLOGY

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ACADEMIC DISSERTATION

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“I will try anything once, twice if I like it, three times to make sure.”
— Mae West
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ABSTRACT

The aim of this study was to characterize novel virulence or fitness genes of *Pectobacterium wasabiae* SCC3193 and *Pectobacterium atrosepticum* SCR11043, which are potato pathogenic model strains. Members of the *Pectobacterium* genus cause plant diseases called soft rot, blackleg and aerial stem rot. Potato is one of the most economically important crops affected by these diseases. The *Pectobacterium* genus belongs to the bacterial family Enterobacteriaceae and is closely related to many common animal pathogens, such as *Escherichia coli* and *Salmonella*. Together, the bacteria belonging to genus *Pectobacterium* and to the other soft-rot genus *Dickeya* are called soft-rot enterobacteria.

In this study, novel virulence genes were identified and characterized using genomic and post-genomic approaches. The study began by examining the genomic sequences of the model strains; functional studies of the genes were then conducted based on function predictions of the genes of interest. The genome of *P. atrosepticum* SCR11043 was sequenced in 2004, and the genomes of *P. wasabiae* SCC3193 and the *P. wasabiae* type strain, which were used for comparative genomics, were sequenced by a collaborator of this study. The genome analyses revised the species designation of the model strain SCC3193 and the genome analyses together with transcriptional studies revealed candidates for novel virulence genes for post-genomic studies. In the post-genomic studies, the genes of interest were mutated and complemented by expression of the respective gene from a plasmid in the mutant background in several phenotypic experiments. The phenotypes of the novel mutant strains were evaluated in several virulence assays in potato and tobacco, motility assays, attachment assays, growth assays in several media, gene expression assays in several media and plant cell wall-degrading enzyme production measuring assays, all in comparison to the wild-type strain and the complemented strain. Plant cell wall-degrading enzymes are the main pathogenicity factors in genus *Pectobacterium*; the other tested functions are known to be related to virulence or may be related to virulence.

Novel virulence determinants were suggested in this study, including the type VI secretion system, the Flp/Tad pilus and a two component-system regulating the pilus, the SirB protein of unknown function and a genomic island containing a predicted lipoprotein transporter and a HopL1-like protein that is similar to the type III secretion system effector HopL1 in *Pseudomonas syringae*. This study provides novel information about the phylogeny and virulence of devastating plant pathogens that are present in Finland and worldwide. This information could be utilized in subsequent applied studies that could enhance plant health as an economically important part of potato production and industry.
LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following original publications that are reprinted and can be found at the end of the review part with the permission of copyright holders:


The publications are referenced in the following text with the respective roman numerals.
ABBREVIATIONS

DNA Deoxyribonucleic acid
Fip Fimbrial low-molecular-weight protein
GI Genomic island
Hcp Hemolysin co-regulated protein
HopL1 Hrp outer protein L1
Hrp Hypersensitive response and pathogenicity
LPS Lipopolysaccharide
PCWDE Plant cell wall-degrading enzymes
qPCR Quantitative polymerase chain reaction
RNA Ribonucleic acid
SAMT Benzoic acid/salicylic acid methyltransferase
SirB Salmonella invasion regulator B
SPI1 Salmonella pathogenicity island 1
T1SS Type I secretion system
T2SS Type II secretion system
T3SS Type III secretion system
T4SS Type IV secretion system
T5SS Type V secretion system
T6SS Type VI secretion system
Tad Tight adherence
TCA Tricarboxylic acid
TCS Two-component system
Tss Type VI secretion system cluster
Vas Virulence-associated secretion
Vic2 Virulence cluster 2

Keywords: Plant pathology, bacteriology, soft-rot enterobacteria, Pectobacterium, virulence, soft rot, blackleg, genomics, plant-microbe interaction, potato production
1 INTRODUCTION

1.1 BACTERIAL SOFT ROT IS A DEVASTATING PLANT DISEASE

Bacterial soft rot afflicts crops and ornamental plants worldwide. The main causative agents of this disease are Gram-negative, rod-shaped, motile and plant cell wall-degrading enzyme (PCWDE)-producing bacteria from the genera *Pectobacterium* and *Dickeya* (formerly known as *Erwinia*). Originally, *Pectobacterium carotovorum* (synonym *Erwinia carotovora*) and *Dickeya* spp. (synonym *Erwinia chrysanthemi*) were characterized as pathogens with broad host ranges: they were reported to infect monocots as well as dicots. However, advanced phylogenetic studies have shown that many strains or species derived from *E. carotovora* and *E. chrysanthemi* display host specificity (De Boer, 2003; Pérombelon, 2002; Yishay *et al.*, 2008). One of the main crops affected by soft-rot enterobacteria is potato (*Solanum tuberosum* L.). Potato is an economically important food and staple crop worldwide. *Pectobacterium* and *Dickeya* belong to the Enterobacteriaceae family, along with many human and animal-associated bacteria, such as *Escherichia coli*, *Salmonella* and *Yersinia*. Investigation of soft-rot enterobacteria could enhance agricultural production and benefit farmers, the potato industry and consumers by providing information about plant health to enhance farming and storage practices.

In potato, three etiologically different types of diseases caused by *Pectobacterium* and *Dickeya* have been described: tuber disease in fields and storage facilities is commonly known as soft rot, mother tuber-borne stem disease is called blackleg and the airborne type of disease is known as aerial stem rot (Czajkowski *et al.*, 2011). Typical bacterial soft rot symptoms in potato tubers include collapsed cell walls, softened creamy or brownish tissue and a distinctive smell. However, typical blackleg and aerial stem rot symptoms are softened and blackish tissue or lesions at the stem base or in upper parts of the stem or leaves (Charkowski *et al.*, 2012; Czajkowski *et al.*, 2011). Disease caused by *Dickeya* species can be indistinguishable from diseases other than soft rot or blackleg due atypical symptoms, such as wilting and tuber vascular rotting, especially in warm climates (Toth *et al.*, 2011).

The best characterized *Pectobacterium* and *Dickeya* species are potato pathogens; the variation in the virulence of these organisms is well known (Ma *et al.*, 2007; Toth *et al.*, 2011). Virulence refers to aggressiveness or symptom severity of a pathogenic microbial strain in comparison to other strains in similar conditions. Typical potato pathogenic *Pectobacterium* species, such as *P. carotovorum*, *P. carotovorum* subsp. *brasiliensis*, *P. atrosepticum* and *P. wasabiae*, are reported to cause soft
rot disease in tubers. However, only \textit{P. carotovorum} subsp. \textit{brasiliensis}, \textit{P. atrosepticum} and \textit{P. wasabiae} are characterized as blackleg pathogens, while \textit{P. carotovorum} is mainly reported in cases of aerial stem rot (Czajkowski \textit{et al.}, 2011). In addition, several \textit{Dickeya} species are found to cause soft rot and blackleg disease (Czajkowski \textit{et al.}, 2011). Currently, the aggressive novel tentative species \textit{Dickeya solani} is spreading vigorously in the Northern hemisphere in much cooler climates than \textit{Dickeya} is commonly found. The biological reason for the higher fitness of \textit{D. solani} in comparison to the traditional potato pathogenic \textit{Pectobacterium} strains in the same area is not clear (Hannukkala, 2011; Toth \textit{et al.}, 2011).

1.1.1 Taxonomy of the soft-rot \textit{Erwinia}

The taxonomical status of the soft-rot enterobacteria \textit{Pectobacterium} and \textit{Dickeya} has been in flux since their first characterizations 1901 and 1953, respectively (Skerman \textit{et al.}, 1980) (Figure 1).

![Figure 1. Evolution of the taxonomy of the potato pathogenic soft-rot \textit{Erwinia}](image)

The genus, species and subspecies status of the soft-rot \textit{Erwinia} is revised several times during years. Nowadays, all the soft-rot \textit{Erwinia} are divided into two genera \textit{Pectobacterium} and \textit{Dickeya}.

In 1945, the genus \textit{Pectobacterium} was described by Waldee, and the species included in this grouping are species we now know as \textit{Pectobacterium} and \textit{Dickeya} (Skerman \textit{et al.}, 1980). However, \textit{Pectobacterium} and \textit{Dickeya} species were classified as \textit{Erwinia} in 1923 and 1953, respectively (Skerman \textit{et al.}, 1980), and this genus has been the prevailing classification in the scientific literature until 1998 when
Hauben et al. (1998) generated three clades from genus *Erwinia* using 16S rDNA sequences. The first clade includes true erwinias, such as *Erwinia amylovora*; the second clade includes soft-rot enterobacteria, namely the *Pectobacterium* genus; and the third clade includes *Brenneria*, which was a novel genus at that time. In 2003 (Gardan et al., 2003), a few subspecies of *P. carotovorum* were raised to the species level; such novel species included *P. atrosepticum*, *P. wasabiae* and *P. betavasculorum*. Recently, a novel species was added to the genus *Pectobacterium*. The *P. carotovorum* strain PC1, whose genomic sequence is available, is now identified as the novel species *P. aroidearum* (Nabhan et al., 2012a). The genus *Dickeya* has a much more recent origin and was organized in 2005 when Samson et al. (2005) separated *P. chrysanthemi* biovars and pathovars into the novel genus *Dickeya*, and the species *D. chrysanthemi*, *D. paradisiaca*, *D. dadantii*, *D. dianthicola*, *D. dieffenbachiae* and *D. zeae* were named.

### 1.1.2 Soft-rot enterobacteria have a worldwide distribution

Soft-rot enterobacteria can be found worldwide from the tropics to boreal zones. The diversity of soft-rot enterobacteria species is expanding worldwide. Recently, a tentative species *D. solani* (biovar 3), a few other *Dickeya* species and *P. wasabiae* were reported as emerging pathogens in potato fields (De Boer et al., 2012; Laurila et al., 2010; Ma et al., 2007; Nabhan et al., 2012b; Ngadze et al., 2010; Pitman et al., 2008; Rezaei & Taghavi, 2010; Stead et al., 2010; Toth et al., 2011; Van Vaerenbergh et al., 2012). Potato plant pathogens are thought to disperse around the world mainly via import and export of seed tubers (Hannukkala, 2011; Toth et al., 2011). Survival and dispersion of soft-rot enterobacteria in potato fields is not completely understood; however, it has been proposed that these organisms survive in crop residues in the soil and can be spread via seed potatoes, agricultural equipment, irrigation and insect vectors such as fruit flies, flies, butterflies or moths (Czajkowski et al., 2011; Perombelon & Kelman, 1980; Toth et al., 2011).

### 1.1.3 Disease outbreaks are common during rainy summers

The genus *Pectobacterium* and *Dickeya* bacteria are necrotrophic pathogens that are traditionally considered to be opportunistic due to their capacity to cause disease in suitable environmental conditions that disable host defenses. Particularly in wet summers, soft rot disease outbreaks are common. In theory, the water film on the surface of a potato tuber creates an anoxic environment in the tuber, inhibiting production of oxygen radicals and enabling multiplication of soft-rot enterobacteria to such levels ($10^7$ cfu/g of plant tissue) that the production of PCWDE is induced and
degradation of the plant cell wall begins rapidly, resulting in typical soft rot symptoms (Pérombelon, 2002). Oxygen radicals have crucial roles in plant defense as stress signal transducers and inducers of local cell death, known as the hypersensitive response, to prevent disease development (Shetty et al., 2008). However, in addition to PCWDE, soft-rot enterobacteria have a wide array of traits that are beneficial in their interactions with plants contributing either to these organisms’ fitness in planta or to virulence (Charkowski et al., 2012).

1.2 SOFT-ROT ENTEROBACTERIA UTILIZE MULTIPLE TRAITS FOR VIRULENCE

1.2.1 Enzymatic disruption of plant cell walls causes typical disease symptoms

The plant cell wall has a complex structure and is composed of cellulose, pectin, hemicellulose and protein. Soft-rot enterobacteria produce massive amounts of specific enzymes that are essential for pathogenesis. These enzymes are secreted through a type II secretion system (T2SS) from the cytosol of the bacteria through the bacterial periplasm into the external milieu or through a type I secretion system (T1SS) from the cytosol to the external milieu, primarily to the plant cell apoplast (Charkowski et al., 2012). In the apoplast, PCWDE disrupt the plant cell wall and liberate nutrients for the consumption of the growing bacterial population. A collection of PCWDE-encoding genes has been characterized in the genus Pectobacterium strains with sequenced genomes, and the vast majority of the pectinases and cellulases were also characterized in previous genetic studies before genome sequencing. PCWDE are reviewed in more detail in (Toth et al., 2003), but a set of putative proteases was identified via genome analyses (Glasner et al., 2008).

1.2.2 Secretion systems play a major role in the transportation of virulence factors

Bacteria have several ways to transport molecules such as enzymes, effector proteins, pilus proteins and heavy metals from the bacterial cytosol to the environment or directly into host cells (Tseng et al., 2009). Bacterial secretion systems play an important role in virulence by injecting virulence factors into the host, and currently, six different types of secretion systems (T1SS-T6SS) are known in Gram-negative bacteria (Tseng et al., 2009) that include potato pathogenic soft-rot enterobacteria in the genus Pectobacterium (Table 1).
Table 1. Secretion systems present in the potato pathogenic *Pectobacterium* species. T1SS=Type I secretion system. T2SS=Type II secretion system. T3SS=Type III secretion system. T4SS=Type IV secretion system. T5SS=Type V secretion system. T6SS=Type VI secretion system. PrtW=Protease W. PCWDE=Plant cell wall-degrading enzymes. Svx=Similar to an avirulence protein from *Xanthomonas*. DspE/F=Disease-specific protein E/F. Cdi=Contact-dependent inhibition. Rhs=Recombination hot-spot. The secretion system is present in the genome (+). The secretion system is not present in the genome (-). The secretion system is present in some of the studied strains (+/-).

<table>
<thead>
<tr>
<th>System</th>
<th>Function</th>
<th>Role in virulence</th>
<th>Studied species</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1SS</td>
<td>Export of PrtW, an adhesin and maybe other proteases and toxins</td>
<td>Required for full virulence</td>
<td><em>P. carotovorum</em> (+) <em>P. carotovorum</em> subsp. <em>brasiliensis</em> (+) <em>P. atrosepticum</em> (+)</td>
<td>Marits et al., 1999; Pérez-Mendoza et al., 2011; Glasner et al., 2008</td>
</tr>
<tr>
<td>T2SS</td>
<td>Export of PCWDE, Svx protein and maybe other virulence determinants</td>
<td>Necessary for pathogenicity</td>
<td>All species (+)</td>
<td>Toth et al., 2003; Glasner et al., 2008; Charkowski et al., 2012</td>
</tr>
<tr>
<td>T3SS</td>
<td>Export of plant physiology modifying T3SS effectors. Only one known effector in <em>Pectobacterium</em> spp. (DspE/F)</td>
<td>Required for full virulence but T3SS is not present in all of the strains</td>
<td><em>P. atrosepticum</em> (+) <em>P. carotovorum</em> (+) <em>P. carotovorum</em> subsp. <em>brasiliensis</em> (+/-) <em>P. wasabiae</em> (-)</td>
<td>Holeva et al., 2004; Pitman et al., 2009; Kim et al., 2009; Kim et al., 2011</td>
</tr>
<tr>
<td>T4SS</td>
<td>Export of unknown molecules</td>
<td>Required for full virulence but T4SS is not widely present in the genus</td>
<td><em>P. atrosepticum</em> (+) <em>P. carotovorum</em> subsp. <em>brasiliensis</em> (+) <em>P. carotovorum</em> (-)</td>
<td>Bell et al., 2004; Glasner et al., 2008; Charkowski et al., 2012</td>
</tr>
<tr>
<td>T5SS</td>
<td>Possibly secretion of large proteins such as serine protease, hemolysin and hemagglutinin. Bacterium-bacterium interaction (Cdi/Rhs)</td>
<td>Not investigated</td>
<td><em>P. carotovorum</em> (+) <em>P. carotovorum</em> subsp. <em>brasiliensis</em> (+) <em>P. atrosepticum</em> (+) <em>P. wasabiae</em> (+)</td>
<td>Glasner et al., 2008; Poole et al., 2011</td>
</tr>
<tr>
<td>T6SS</td>
<td>Export of unknown T6SS effectors</td>
<td>Required for full virulence and may be related to microbe-microbe interaction</td>
<td><em>P. atrosepticum</em> (+)</td>
<td>Liu et al., 2008; Mattinen et al., 2008</td>
</tr>
</tbody>
</table>

The T2SS and T1SS are related to the export of virulence factors proximal to the host cells. In soft-rot enterobacteria, the T2SS is the main secretory system for PCWDE responsible for pathogenesis and is also utilized for the secretion of other virulence proteins such as Svx, a protein similar to an avirulence protein from *Xanthomonas* (Charkowski et al., 2012; Corbett et al., 2005). The Finnish model strain *Pectobacterium* sp. SCC3193 expresses an important T1SS-secreted protease (PrtW) that is necessary for full virulence in potato (Marits et al., 1999). PrtW is highly similar to...
proteases characterized in *Dickeya* (PrtB, PrtC, PrtA, PrtG) but not to other previously characterized secreted proteases of *Pectobacterium* species (Marits et al., 1999). A T1SS-secreted adhesin has also been shown to contribute to the virulence of *P. atrosepticum* (Pérez-Mendoza et al., 2011a). The type III secretion system (T3SS) is a well-studied effector protein translocation system targeting host cells that is important in virulence in many biotrophic or hemibiotrophic plant pathogens (Büttner & He, 2009). The type III secretion system is notorious for its effectors, which have been found to modulate host gene expression, affecting host physiology and suppressing plant defense. Among plant pathogenic bacteria, the hemibiotrophic *Pseudomonas syringae* may be the best-studied model for T3SS research. In *P. syringae*, many T3SS effectors have been identified that modulate host physiology to favor bacteria (Lindeberg et al., 2012). The T3SS is also a subject of study in soft-rot enterobacteria. In *Dickeya*, this secretion system has important roles related to virulence in infections of African violet and in pellicle formation (Yang et al., 2002; Yap et al., 2005). In *Pectobacterium* species, the T3SS has a modest role in virulence and is suggested to be important during the early stages of infection (Holeva et al., 2004; Kim et al., 2011). It is also possible that some *P. carotovorum* and *P. wasabiae* strains lack the T3SS that is present in other soft-rot enterobacteria (Kim et al., 2009; Pitman et al., 2009). In soft-rot enterobacteria, only a handful of effectors have been identified, and only one effector (DspE/F) from *P. carotovorum* has been shown to have a function in cell death but not in the induction of other defense reactions, such as callose formation (Kim et al., 2011). The type IV secretion system (T4SS) has most likely been best studied in *Agrobacterium tumefaciens*, which utilizes a T4SS to translocate the main virulence factors (transfer DNA, carrying plant hormone and opine synthesis genes) that generate the typical tumors that establish symptoms of crown gall (Pitzschke & Hirt, 2010). It is not known what role the T4SS plays in soft-rot enterobacteria, but it has been shown that this secretion system is needed for full virulence in potato tubers (Bell et al., 2004). Type V secretion systems (T5SS) have not been found to be related to virulence of soft-rot enterobacteria. Most recent addition into the secretion system continuum is type VI secretion system (T6SS) that has been studied also in *Pectobacterium*. It is shown that T6SS is needed for full virulence in *P. atrosepticum* in potato (Liu et al., 2008). No secreted effectors via type VI secretion system are known in soft-rot enterobacteria but it is likely that they will be characterized in the future and very promising candidates can be found associated to the core cluster encoding the secretion system machinery (Liu et al., 2008).
1.2.3 Motility, attachment and biofilm formation enhance plant colonization

Flagella based motility is considered to be necessary for full virulence of soft-rot enterobacteria (Antúnez-Lamas et al., 2009; Hossain et al., 2005; Mulholland et al., 1993; Pirhonen et al., 1991). Bacteria have also other mechanisms of movement, such as the type IV pili (Jarrell & McBride, 2008), but thus far, no one has reported the function of type IV or any other pili in the motility or virulence of soft-rot enterobacteria. Attachment is an important virulence determinant in many Gram-negative pathogenic bacteria. In soft-rot enterobacteria, the role of attachment is not well-studied, although attachment may be a very important function for host colonization and survival in the soil or plant residues. *P. carotovorum* flagella are needed for attachment to artificial surfaces and for virulence in potato (Hossain & Tsuyumu, 2006). In *Dickeya*, the T3SS is necessary for the production of pellicles that are biofilm-like cell formations floating on the surfaces of liquid bacterial cultures (Yap et al., 2005). An adhesin in *P. atrosepticum* is needed for efficient attachment to plant roots and for full virulence in potato stems (Pérez-Mendoza et al., 2011a). It is known that *P. atrosepticum* generates biofilms poorly in *in vitro* conditions and that attachment is likely tightly regulated. However, in a few cases, genetically modified strains of *P. atrosepticum* SCRI1043 have been shown to produce more biofilm than the wild-type strain. Mutagenesis of essential lipopolysaccharide (LPS) synthesis genes (*rffG* solely and *rffG* together with *waaJ*) significantly increases biofilm formation (Evans et al., 2010), and overproduction of the c-di-GMP signaling molecule *in trans* increases biofilm formation and translocation of a biofilm adhesin polysaccharide (poly-β-1,6-N-acetyl-D-glucosamine) that is necessary for wild-type levels of biofilm formation (Pérez-Mendoza et al., 2011b).

1.2.4 Other plant-targeting toxins and the biochemical battle within plant tissue

Soft-rot enterobacteria can also produce secondary metabolites that act as phytotoxins. *P. atrosepticum* SCRI1043 carries *cfa* genes encoding proteins needed for the production of coronafacic acid, which is a compound of the phytotoxin coronatine, an important *P. syringae* virulence factor, and *syr* genes for syringomycin, which is a membrane pore-forming phytotoxin in *P. syringae* pv. *syringae* (Bell et al., 2004). It is likely that coronafacic acid itself also acts as a phytotoxin, as the mutagenesis of the *cfa* gene significantly decreases *P. atrosepticum* virulence (Bell et al., 2004). Recently, other secondary metabolites besides host-targeting toxins have been shown to play roles in the virulence of soft-rot enterobacteria. Soft-rot enterobacteria are able to modify the biochemical conditions within a plant, enabling survival and disease establishment. The butanediol
production pathway is conserved in soft-rot enterobacteria, and the production of butanediol has an important role in disease development during the maceration stage (Effantin et al., 2011). Butanediol production is necessary for the successful modification of pH that contributes to the alkalinization of plant tissue, enhancing the activity of PCWDE (Effantin et al., 2011; Marquez-Villavicencio et al., 2011). Mutagenesis of an essential butanediol pathway gene in the *bud* operon decreases virulence of both *P. carotovorum* and *D. dadantii* in potato tubers (Effantin et al., 2011; Marquez-Villavicencio et al., 2011). Citric acid, the first product in the tricarboxylic acid (TCA) cycle, is present in large quantities in plant tissue. In *P. atrosepticum*, a citric acid transporter responsible for the uptake of citric acid decreases the citric acid concentration in infected tissue and is necessary for full virulence in potato tubers (Urbany & Neuhaus, 2008). The function of citric acid uptake in virulence is not known, but it is speculated to be related to citric acid-dependent achromobactin siderophore synthesis, the function of the bacterial TCA cycle in anoxic conditions or the disruption of TCA cycle-dependent reactive oxygen species homeostasis (Urbany & Neuhaus, 2008). Iron acquisition from the extracellular milieu is important for microbes, and many families of siderophores (iron-chelating secreted compounds) are utilized to acquire iron from the bacterial cell’s surroundings. Siderophore synthesis genes are present in both *Pectobacterium* and *Dickeya*, and both genera have been shown to produce siderophores (Bell et al., 2004; Bull, 1996). It has only been shown that siderophore production has a significant impact on virulence in *Dickeya* (Franza et al., 2002). *D. dadantii* also produces the essential plant hormone auxin (*iaa* genes), and mutagenesis of the *iaa* gene has a significant effect on virulence in the African violet (Yang et al., 2007). Based on the knowledge available today, only *Dickeya*, and not *Pectobacterium*, has been shown to be able to produce auxin (Charkowski, 2009).

### 1.2.5 Genes necessary for bacterium-insect or bacterium-bacterium interactions

Soft-rot enterobacteria may also have traits that are necessary for bacterium-insect or worm interactions, bacterium-bacterium interactions, or bacterium-fungus interactions, which may play a role in the ecology of *Pectobacterium* and *Dickeya*. Insects or worms, such as nematodes, can ingest bacteria as food or can act as vectors for the dispersion of bacteria. Soil, the rhizosphere and plant tissue contain a number of microbial species that could be beneficial or harmful to plants or animals and that compete with soft-rot enterobacteria for vital resources. Many *P. carotovorum* strains carry an *evf* gene that encodes an S-palmitoylated protein that is necessary to allow bacterial utilization of fruit flies (*Drosophila melanogaster*) as vectors by attaching bacteria to lipid vesicles.
(Basset et al., 2003; Quevillon-Cheruel et al., 2009). D. dadantii carries cyt genes that produce a pore-forming toxin, causing septicemia in pea aphids (Acyrthosiphon pisum) (Costechareyre et al., 2012; Grenier et al., 2006). Bacteria can use several traits to recognize other bacteria in the same niche, and many species also produce a range of toxins to eliminate competing bacterial species. Dickeya and P. wasabiae carry contact-dependent inhibition (Cdi) and Rhs (recombination hot-spot) family toxin/immunity systems, which inhibit the growth of E. coli lacking the corresponding immunity gene (Aoki et al., 2010; Poole et al., 2011). Cdi and Rhs toxin/immune systems are likely membrane-spanning T5SS-like proteins, secreting the toxic tip of the membrane protein (Aoki et al., 2010; Charkowski et al., 2012; Poole et al., 2011). A classic example of an antibiotic produced by several soft-rot enterobacteria species is carbapenem, which is synthesized in parallel to PCWDE in response to a cell quorum (Barnard & Salmond, 2007). Soft-rot enterobacteria also have genes for other bacteriocins in strain- or species-specific manners (Glasner et al., 2008). The phage tail-like bacteriocin (carotovoricin) has been characterized in more detail in P. carotovorum (Nguyen et al., 2001). Recently, two novel colicin M-like bacteriocins (pectocin M1 and pectocin M2) were identified in P. aroidearum and P. carotovorum subsp. brasiliensis, which target other Pectobacterium species (Grinter et al., 2012).

1.2.6 Regulation of virulence determinants is coordinated

Soft-rot enterobacteria encounter a hostile environment in planta, and very little is known about how these microorganisms evade plant defense during latent infection. However, it is thought that the timing of maceration is an important step to avoid plant defense. Degradation products of the plant cell wall induce plant defenses (Norman et al., 1999; Norman-Setterblad et al., 2000). In addition, the T3SS effector HrpN and a PCWDE, polygalacturonase (PehA) of P. carotovorum can induce systemic resistance in plants (Kariola et al., 2003). Utilization of multiple virulence determinants in soft-rot enterobacteria is orchestrated via a complex regulatory network to time the virulent stage relative to the best environmental conditions and host physiology (Charkowski et al., 2012).

Based on current knowledge, the center of the regulatory network in soft-rot enterobacteria is the switch-like RsmA-RsmB-RsmC system, which coordinates signal transduction to manage the expression of virulence-related genes (Charkowski et al., 2012). RsmA is a transcriptional regulator that functions as a repressor of virulence genes in unfavorable conditions (Charkowski et al., 2012). In addition to PCWDE and flagella, it has been recently shown that RsmA likely also regulates several other genes, including the type III secretion system, the type VI secretion system and genes used in butanediol fermentation (Kõiv et al., 2013). The rsmB-gene
encodes a post-transcriptional regulatory RNA that functions as a repressor of RsmA in response to environmental stimuli, such as compounds from degraded plant tissue, as a signal to start the production of PCWDE (Charkowski et al., 2012). RsmC is a repressor of the main flagellar regulator FlICD complex, thus affecting flagella biogenesis and the production of PCWDE (Charkowski et al., 2012). Another crucial switch-like system in soft-rot enterobacteria is a population density-dependent quorum-sensing system. Quorum sensing is a common social gene regulatory mechanism that is based on the concentration of small N-acyl-homoserine lactone (N-acyl-HSL) molecules secreted by a population (Barnard & Salmond, 2007; Põllumaa et al., 2012). Individual bacterial cells produce auto-inducer molecules whose concentration increases and affects virulence only when the population density of soft-rot enterobacteria reaches a minimum of $10^7$ cells per gram of plant tissue (Pérombelon, 2002). Within soft-rot enterobacteria, five forms of quorum-sensing molecules are known: 3-oxo-C6-HSL, 3-oxo-C8-HSL, 3-oxo-C10-HSL, C6-HSL and C8-HSL (Crépin et al., 2012). Within soft-rot enterobacteria, many strains produce two or three different forms of HSLs; however, commonly, only one of the isoforms dominates (Barnard & Salmond, 2007; Crépin et al., 2012). A huge proportion of the genes of soft-rot enterobacteria are regulated by quorum sensing, including the characterized regulation of the production of PCWDE, which were one of the earliest targets in the study of the genetics of soft-rot enterobacteria (Barnard & Salmond, 2007; Liu et al., 2008; Põllumaa et al., 2012). Soft-rot enterobacteria strains in which the quorum system gene or genes are mutated are generally found to be avirulent in planta, with the exception of some Dickeya strains in which quorum sensing is less important in virulence, though the quorum does regulate pectinase production (Barnard & Salmond, 2007; Crépin et al., 2012; Põllumaa et al., 2012).

In addition to these switch-like systems that affect multiple traits, bacteria also have cell wall-binding sensors, such as kinases of two-component systems (TCS) and the Rcs phosphorelay system, which mediate signals to the corresponding response regulator regulating a specific operon or gene set in response to environmental stimuli. In soft-rot enterobacteria, several two-component systems, such as ExpA-ExpS, PehR-PehS and PmrB-PmrA, also regulate virulence in response to environmental cues (Charkowski et al., 2012). The Rcs phosphorelay system (RcsA-RcsB-RcsC) is known to regulate virulence as a repressor in Pectobacterium (Andresen et al., 2007, 2010). Other types of sensors in the cytosol are encoded as one-component sensors that are known to sense the environment via molecules that have passed through the cell wall. Known one-component systems in soft-rot enterobacteria include ExpR, which senses the elevation of quorum-sensing molecules and activates PCWDE-encoding genes, and KdgR and HexA, which are transcriptional repressors of PCWDE (Charkowski et al., 2012; Nasser et al., 1992).
2 AIMS OF THE STUDY

The focus of this study was to investigate novel virulence-related genes of potato pathogenic soft-rot enterobacteria of the *Pectobacterium* genus. I utilized a genomic approach consisting of computational methods to investigate gene functions and post-genomic approach involving applications of other methods to investigate hypotheses rising from genomic information to understand better the genetic background of bacterial soft rot and provide information for applied studies to control this disease and enhance agriculture, food industry and food security. The study can be divided into three sub-sections:

1. Evaluation of the species status of the model *Pectobacterium* strain SCC3193
2. Identification of novel virulence determinants from genomic sequence
3. Experimental characterization of novel virulence determinants
3 MATERIALS AND METHODS

In this study, a diverse methodology was utilized to investigate the phylogeny, virulence and gene regulation of soft-rot enterobacteria of the *Pectobacterium* genus (Table 2).

Table 2. Summary of methods and model organisms used in this study. DNA=Deoxyribonucleic acid. PCR=Polymerase chain reaction. RNA= Ribonucleic acid.

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4 RESULTS AND DISCUSSION

4.1 GENOMIC APPROACH TO TAXONOMY AND VIRULENCE DETERMINANTS OF PECTOBACTERIUM

4.1.1 Taxonomic positions of the model strains SCC3193 and SCR1043

The taxonomic grouping of soft-rot enterobacteria has been changing constantly since the first characterizations of these plant pathogenic bacteria in the early 20th century. The most recent changes in this taxonomy are the revised classification of plant pathogenic enterobacteria (family Enterobacteriaceae), in which all soft-rot enterobacteria were classified into the genus *Pectobacterium* (Hauben *et al.*., 1998), and the division of *Pectobacterium* into the two genera *Pectobacterium* and *Dickeya* a few years later (Samson *et al.*, 2005). The soft-rot enterobacteria model strains *Pectobacterium* sp. SCC3193 and *Pectobacterium atrosepticum* SCR1043 utilized in this study (I; II; III; IV) are both devastating potato pathogens causing soft rot and blackleg in potato. Both strains are isolated from potato stem and are relatively well studied, and both strains’ genomes have been sequenced (II; Bell *et al.*, 2004), enabling the use of post-genomic methodology for the phylogenetic analyses of soft-rot enterobacteria and the characterization of novel virulence-related traits and regulatory pathways in this study.

One of the main findings of this study was that the model strain SCC3193 is a member of the novel species *P. wasabiae* (species status in 2003 by Gardan *et al.*) based on genomic sequence (II). In the original publication describing SCC3193 in 1988, this organism was characterized as a subspecies of *P. carotovorum* (*P. carotovorum* subsp. *E. carotovora* subsp. *carotovora*). The species *P. wasabiae* (*E. carotovora* subsp. *wasabiae*) was first characterized around the same time as SCC3193 and was published in 1987 in Japan (Goto & Matsumoto, 1987). Suspicions regarding the species designation of SCC3193 arose after the investigation of the genome, when high levels of sequence similarity and open reading frame (ORF) synteny with the previously sequenced *P. wasabiae* WPP163 (NC_013421.1; Kim *et al.*, 2009) were identified (I; II). Addition of the genomic sequence of the *P. wasabiae* type strain into the analysis confirmed the species designation of SCC3193 (II). However, differences in the genome sequence, traits identified in the genome and host preference suggest that the *P. wasabiae* clade isolated from potato may represent a novel subspecies or even a novel species, although more work is necessary to confirm the hypothesis (II). The revision of the species designation of SCC3193 had to wait until the genomic era, likely
because of the wide variation within the *P. carotovorum* species that has been previously noted and because the differentiation between *P. wasabiae* and *P. carotovorum* strains isolated from potato has been difficult. Overall, the determination of bacterial species is difficult, and the taxonomic classification of many bacterial groups is constantly changing; soft-rot enterobacteria are no exception.

Interestingly, *P. wasabiae* and *P. atrosepticum* are closely related based on both phylogenetic analyses and the comparison of the strain proteomes (II). This suggests that these species could provide a window into mechanisms of how some soft-rot enterobacteria are restricted to the tuber, causing classical soft rot, and how some species (mainly *P. wasabiae, P. atrosepticum, P. carotovorum* subsp. *brasiliensis* and some species of *Dickeya*) are able to disperse from the tuber to the stem, causing classical blackleg. Previously, Glasner and colleagues (2008) utilized genomic information to determine the niche specificity of *Pectobacterium* in comparison with *P. carotovorum*, *P. atrosepticum* and *P. carotovorum* subsp. *brasiliensis*, but no clear result was obtained as to why these bacteria have different niches. Overall, the investigated strains differed in many traits, such as toxin production, enzyme production, antibiotic production, gene regulation and metabolism (Glasner et al., 2008). Perhaps when more soft-rot enterobacteria from different niches have had their genomes sequenced, it will be possible to associate specific traits with niche specificity. However, it is also possible that niche specificity has evolved as combinations or deletions of multiple traits.

Recently, the species *P. wasabiae* has been observed as an emerging pathogen in potato fields in Europe, the Middle East, United States, New Zealand and South Africa (Baghaee-Ravari et al., 2011; Dung et al., 2012; Ma et al., 2007; Moleleki et al., 2013; Nabhan et al., 2012b; Pitman et al., 2008). Based on our work (SCC3193 was isolated in the early 1980s, I; II), it is not clear whether the potato-related *P. wasabiae* is an emerging pathogen or a group of bacteria that were incorrectly characterized before the genomic era due to the lack of distinctive methodology to separate *P. carotovorum* and *P. wasabiae* isolated from potato. However, *P. wasabiae* has also been reported to be an emerging pathogen in Korean Japanese horseradish fields (Kim et al., 2012). It is likely that some of the novel findings in potato fields are due to seed potato import and export, as the sequence similarity of the first sequenced *P. wasabiae* from the United States was largely identical to our Finnish isolate *P. wasabiae* SCC3193 and differed considerably from the type strain based on genomic sequence (II). One option is that some environmental or agricultural factors have changed in addition to seed potato export/import, enhancing the dispersion and survival of *P. wasabiae*. Perhaps both emerging soft-rot enterobacteria *P. wasabiae* and *D. solani* (Toth et al., 2011; II) are faring better in specific bacterium-bacterium interactions compared with traditional *Pectobacterium* strains and *Dickeya*. Alternatively, both emerging species may prefer similar environmental
conditions, enabling the rise of both species in potato fields and allowing them to occupy the niches of other *Pectobacterium* species. However, more work is needed to examine the reasons for the rise of these two species in potato fields.

4.1.2 Virulence and fitness-enhancing traits are in the core and variable genomes of *Pectobacterium*

Genome sequences have been used to characterize novel virulence-related genes or genes related to other beneficial traits for the ecology of soft-rot enterobacteria and several other bacterial species. The first genomic sequence of soft-rot enterobacteria, the genome of *P. atrosepticum* SCRI1043, was published in 2004 (Bell *et al*., 2004). Based on this sequence, a comparative genomic analysis was published two years later in which the differences and similarities between the plant and animal pathogenic enterobacteria were compared (Toth *et al*., 2006). In 2008, the first comparative genomics study between *Pectobacterium* was published, attempting to explain the niche specificities of soft-rot and blackleg bacteria (Glasner *et al*., 2008). In the present study (II; III; IV), genomic information was utilized to investigate novel virulence determinants in *Pectobacterium*, specifically in the long-term model strain SCC3193 (Figure 2).

As in most bacterial species, the genome of soft-rot enterobacteria can be divided into the core genome and the variable genome. The core genome is considered to be an ancient part of the genus or species that shows the evolutionary origin of the bacterial group (Juhas *et al*., 2009). The core genome commonly contains traits that are necessary for the viability and fitness of the bacteria (Juhas *et al*., 2009). The variable genome contains additional traits that benefit the strain or species in specific environments or in interactions with specific foes (Juhas *et al*., 2009). The variable genome is often composed of island-like structures, often called genomic islands (GIs/GEIs), horizontally acquired islands (HAIs) or pathogenicity islands (PAIs). These areas of the genome can be characterized based on differing sequence composition (such as GC% in comparison to the surrounding genome) or due to the lack of the island in other close relatives (Dobrindt *et al*., 2004; Hacker & Carniel, 2001; Juhas *et al*., 2009; Toth *et al*., 2006). These islands are usually considered to be acquired by horizontal gene transfer and adopted from an organism that does not share the same evolutionary origin. It is also thought that these traits are commonly highly beneficial for the receiving bacteria (Dobrindt *et al*., 2004; Hacker & Carniel, 2001; Juhas *et al*., 2009).
Figure 2. Novel and known virulence and fitness related traits in the potato pathogenic *Pectobacterium wasabiae* strains SCC3193 and WPP163 (II). These strains share many traits that are rare or absent from other *Pectobacterium* strains and *P. wasabiae* type strain CFBP 3304T. Such traits are for example SAMT, FerE, Cdi, LPS loci composition, aerobactin and T4 pili. SirB=Salmonella invasion regulator B-like protein. T6SS=Type VI secretion system. Vic2/HopL1=Virulence cluster 2 that contains T3SS effector-like gene hopL1. T3SS=Type III secretion system. LPS=Lipopolysaccharide. PCWDE=Plant cell wall-degrading enzymes. Svxx=Similar to an avirulence protein from *Xanthomonas*. T2SS=Type II secretion system. Cdi=Contact-dependent inhibition. Rhs=Recombination hot-spot. T1SS=Type I secretion system. PrtW=Protease W. T4=Type IV. Nip=Necrosis inducing protein. Flp=Fimbrial low-molecular-weight protein. BMC=Bacterial micro-compartment. T4SS=Type IV secretion system. SAMT=Benzoic acid/salicylic acid methyltransferase. Vic1/Type I R-M system=Virulence cluster 1 that contains type I restriction-modification system. FerE=Ferredoxin-like protein.

To investigate novel virulence determinants in *Pectobacterium*, our focus was on investigation of genomic islands and other novelties found from the genome (II; III; IV). Most of the previously described virulence determinants of *Pectobacterium* are present in the core genome of SCC3193 (II) with the exception of the Nip toxin-encoding gene, which induces necrosis in planta (Mattinen et al., 2004; Pemberton et al., 2005). Other exceptions are the genes encoding the ferredoxin-like protein FerE, which has been found to enhance survival in oxidative stress and in planta (Sjöblom et al., 2008), and DsbA, which modifies secreted proteins, including PCWDE, and is, necessary for virulence (Coulthurst et al., 2008; Vincent-Sealy et al., 1999). Other putative novel virulence determinants were characterized from the core genomes of *P. wasabiae* SCC3193 and *P. atrosepticum* SCHR043 in this study (II; III; IV). One determinant was the T6SS-encoding cluster.
(T6SS-1 in SCC3193), which encodes a putative plant modifying effector secretion system and is an important virulence determinant in many animal pathogens (Records, 2011). A second determinant was the Flp/Tad pilus-like cluster, which may be necessary for attachment or biofilm formation based on research in animal pathogens and one potato pathogen (Tomich et al., 2007; Wairuri et al., 2012). A third determinant is the predicted virulence regulator SirB, which may be related to the regulation of virulence in *Salmonella*, although no clear function has yet been demonstrated in *Salmonella* or in *E. coli* (Johnston et al., 1996; Rakeman et al., 1999; Strohmaier et al., 1995). The genes in the same cluster as SirB have important cellular functions in *E. coli* related to LPS synthesis and protein translation (Nakahigashi et al., 2002; Ryden et al., 1986; Strohmaier et al., 1995).

During the GI analyses, it was clear that prediction was not an easy task due to the ambiguous nature of the islands. Thus, we decided to combine three computational methods (thanks to the bioinformatician Mr. Koskinen) and manually evaluate islands to characterize GIs in SCC3193 (II). Many of these islands were highly specific to SCC3193 or to SCC3193 and other sequenced *P. wasabiae* strains among soft-rot enterobacteria. These islands contain putative virulence determinants, such as a second T6SS cluster (T6SS-2), which is typically encountered in species of genera *Erwinia* and *Pantoea*, phage-related genes and the type I site-specific restriction-modification system-containing island (virulence cluster 1; Vic1) that is related to virulence in *Yersinia*, an island containing a putative protein transportation system and the T3SS effector HopL1-like protein (virulence cluster 2; Vic2), and a putative benzoic acid/salicylic acid methyltransferase (SAMT) that could have an effect on defense signaling in plants (II). Many novel traits were also identified that related to microbe-microbe competition, such as several putative bacteriocin-encoding clusters and contact-dependent growth inhibition (Cdi) loci (II). Additionally, novel traits that may benefit SCC3193 *in planta* or in the environment were identified; such traits included a wide range of surface structure-related clusters (membrane proteins, lipopolysaccharides and lipooligosaccharides), a novel bacterial microcompartment that could be related to anaerobic utilization of an unknown carbon source, an arsenate resistance cluster and an aerobactin siderophore locus for iron acquisition (II).

The island prediction process has its own difficulties, and more specific characterization of these islands is needed. Many of the islands predicted in this study (II) are commonly present in soft-rot enterobacteria. Some of these islands were either predicted to be GIs in other *Pectobacterium* strains or to contain genes such as phage related genes that are considered to be signs of horizontal gene transfer, even though the transfer may have occurred in the dawn of evolution and the trait, such as flagella, is now commonly present in the majority of known bacterial species (II). Despite the problems with the accuracy of the GI prediction, this model seemed to provide a beneficial tool to analyze novel traits in a
newly sequenced species and to possibly identify niche-specific traits. In this study, the genomic island analyses provided an interesting view of the long-term soft-rot enterobacteria model strain \textit{P. wasabiae} SCC3193, raising a number of hypotheses for post-genomic studies and for the collection of empirical evidence supporting the predicted novel virulence determinants.

\section*{4.2 POST-GENOMIC APPROACH TO CHARACTERIZE NOVEL VIRULENCE DETERMINANTS OF \textit{PECTOBACTERIUM}}

\subsection*{4.2.1 Type VI secretion system may mediate interactions with the host}

The type VI secretion system is a widespread protein secretion system in Gram-negative bacteria, particularly Proteobacteria. The T6SS is encoded by a T6SS core cluster that is commonly composed of a minimum of 13 conserved genes and additional \textit{hcp-vgrG} islands that can carry effectors and are scattered around the genome (reviewed recently in Coulthurst, 2013; Kapitein & Mogk, 2013). The function of the T6SS has been studied extensively in animal pathogenic bacteria where this system has been found to allow microorganisms to target eukaryotes, host or small eukaryotic predators in the environment, and prokaryotes competing in the same niche (Coulthurst, 2013; Kapitein & Mogk, 2013). In plant-associated bacteria, the function of the T6SS is not as well studied as in animal pathogens. In many cases, the effectors or functions of the effectors are not known, providing an opportunity for future studies of the T6SS in phytopathogens.

The function of the T6SS has been identified in only in a few plant pathogenic bacteria and one N-fixing plant symbiotic bacterium. In \textit{P. syringae}, the T6SS has been investigated as a host-targeting system, but no clear evidence of such a system has been presented; instead, the T6SS of \textit{P. syringae} was shown to be necessary for competition with \textit{E. coli} and to suppress the growth of yeast and other competitors in the same niche (Haapalainen \textit{et al.}, 2012). In \textit{A. tumefaciens}, the T6SS is related to symptom severity and mutagenesis of the essential T6SS gene \textit{hcp} that encodes the likely syringe part of the secretion system. This cluster reduces gall formation in a potato tuber disk model, but the mutagenesis of VasK/TssM or the T6SS cluster did not affect gall formation (Wu \textit{et al.}, 2008), leaving questions open concerning the role of the T6SS in virulence of \textit{A. tumefaciens}. VasK has sequence similarity to the type IVb secretion system membrane protein IcmF, and VasK interacts with the IcmF structure-stabilizing protein VasF/TssL (IcmH/DotU family) and functions as an ATPase, providing energy for Hcp secretion in the T6SS system (Ma...
et al., 2012). In *Rhizobium leguminosarum* bv. *trifolii*, the T6SS is necessary for host specificity and for the secretion of proteins responsible for this specificity such as RbsB, which is similar to ribose-binding proteins in *Bacillus subtilis* and *Vibrio cholerae* (Bladergroen et al., 2003; Roest et al., 1997).

In soft-rot enterobacteria, the T6SS is related to microbe survival *in planta* and may be directly related to virulence. In this study, early experiments with T6SS in *P. atrosepticum*SCRI1043 indicated that the T6SS is expressed in response to plant extracts and plant tissue and could be related to the suppression of virulence due to the hypervirulence that followed mutagenesis of the ECA3435 (VasH) or ECA3432 (VasK) genes within the core T6SS cluster (III). ECA3435 encodes the sigma 54-dependent transcriptional activator VasH, which is the transcriptional regulator of the *hcp* and *vgrG* genes, enabling the co-regulation of the core T6SS cluster and scattered genome-wide *hcp-vgrG* islands (Bernard et al., 2011). Interestingly, at the same time Liu et al., (2008) published an opposing phenotype for T6SS mutant strains of *P. atrosepticum*SCRI1043, in which mutagenesis of the essential T6SS components ECA3438 (VasE/TssK) or ECA3444 (VipB/TssC) decreases virulence in potato tubers and stems. ECA3438 is similar to the *Vibrio cholerae* VCA0114, which is necessary for a functional T6SS although the exact function is unknown (Zheng et al., 2011). ECA3444 is similar to the VipB protein that is necessary for T6SS-dependent secretion of *V. cholerae* V52. This protein interacts with VipA to form a cogwheel-like structure and with ClpV (AAA+ ATPase) to provide energy for the function of T6SS (Bönemann et al., 2009). Later, it was found that the phenotype of *Pectobacterium* T6SS mutants could be dependent on the virulence assay used. With low cell concentrations as in (Liu et al., 2008), the *P. atrosepticum* ΔvasH utilized in our study (III) and a *P. atrosepticum*SCRI1043 strain lacking the T6SS gene cluster (ΔT6SS) displayed delayed symptom development in comparison to the wild-type strain (J. Nykyri, unpublished). The extraordinary phenotype of the highly hypervirulent *P. atrosepticum* ΔvasK (III) was a puzzle, but after phage transduction of this mutation onto clean background, this mutant’s phenotype was parallel to ΔvasH and ΔT6SS, indicating that the original ΔvasK mutant had secondary mutations affecting its phenotype (J. Nykyri, unpublished). At the time of the investigation of the T6SS clusters in *P. wasabiae* SCC3193, a different virulence assay was utilized that aimed for easily controllable experiments and measurements, and potato tuber slices provided an ideal model for that purpose (Sjöblom et al., 2008). The mutagenesis of T6SS-1 or T6SS-2 in *P. wasabiae* SCC3193 did not affect disease development, but mutagenesis of both T6SS clusters delayed symptom formation, indicating that the T6SS clusters are at least partially redundant and that the decreased virulence was a consequence of the mutated clusters (II). The T6SS clusters in *P. wasabiae* likely have different origins. The T6SS-1 cluster is similar to the corresponding cluster in other
soft-rot enterobacteria, but the T6SS-2 cluster is more similar to clusters present in the *Erwinia* and *Pantoeae* genera, indicating that the T6SS-2 was likely acquired horizontally in *P. wasabiae* (II). The regulation of the T6SS in soft-rot enterobacteria has been at least partially characterized in recent years. This regulation is known to be mediated parallel (quorum sensing, RsmA) to major virulence determinants in response to plant tissue, plant mimicking conditions (potato tuber extract) or the commercially available plant cell wall compound polygalacturonic acid (Liu *et al.*, 2008; Mattinen *et al.*, 2007; III; IV; J. Nykyri, unpublished).

So far, all of the T6SS mutant strains of *Pectobacterium* show only slight, but statistically significant, effects on disease development. However, the T6SS is expressed and regulated similarly to major virulence determinants, indicating that the T6SS likely does not secrete major virulence factors but may be otherwise related to fitness *in planta*. The virulence assay conditions may not fully display the functions of the T6SS, the effectors may have a minor effect on the host or the T6SS may be necessary for competition with the natural microbial flora in potato. However, preliminary experiments to study the T6SS of *Pectobacterium* in bacterium-bacterium (*D. solani*, *E. coli*, *P. syringae*) or bacterium-fungi (*Rhizoctonia solani*) interactions were performed, but no evidence was gathered that the T6SS protects *Pectobacterium* from microbial competitors (J. Nykyri, unpublished). Perhaps a wider screen of organisms is needed to identify the target of the T6SS in *Pectobacterium*, or perhaps the target is indeed the host plant, and further investigations of effectors will reveal a fundamental function of the T6SS in *Pectobacterium*.

### 4.2.2 Flp/Tad pili could play a role in attachment or biofilm formation during maceration

The Flp/Tad pilus is a type IVb pilus that is commonly related to attachment or biofilm formation and virulence/host colonization. The Flp/Tad pilus is encoded by the *flp/tad* gene cluster (*flp-rcp-tad*), which is composed of 13 conserved genes, some of which are required, while others not necessary for the production of functional pili (Tomich *et al.*, 2007). The Flp/Tad pilus is a very well characterized virulence determinant in *Aggregatibacter actinomycetemcomitans*, the causative agent of localized aggressive periodontitis (Bhattacharjee *et al.*, 2001; Clock *et al.*, 2008; Kachlany *et al.*, 2001; Kram *et al.*, 2008; Perez *et al.*, 2006; Tomich *et al.*, 2006). The Flp/Tad pilus is also characterized in other animal pathogens, such as *Yersinia enterocolitica*, *Pseudomonas aeruginosa*, *Haemophilus ducreyi* and *Pasteurella multocida* (Bernard *et al.*, 2009; Harper *et al.*, 2003; Nika *et al.*, 2002; Schilling *et al.*, 2010). The Flp/Tad pilus is widely dispersed among bacterial species and has also been characterized in the environmental bacterium *Caulobacter crescentus* (Skerker & Shapiro, 2000), in the human gut bacterium *Bifidobacterium*
breve and in the potato pathogen *Ralstonia solanacearum* (O’Connell Motherway *et al*., 2011; Wairuri *et al*., 2012). *R. solanacearum* is the only plant pathogen in which the Flp/Tad pilus has been characterized, and it contributes to virulence in the potato and to biofilm formation (Wairuri *et al*., 2012). For unknown reasons, the mutagenesis of the *flp/tad* gene (TadA2; NTPase) in *R. solanacearum* increased the biofilm production instead of decreasing it, which is an opposing phenotype compared to previous studies on other bacteria (Wairuri *et al*., 2012).

In the present study (III), we obtained results suggesting that the Flp/Tad pilus could be an important surface structure in *Pectobacterium* during host colonization. The *flp/tad* genes of *P. atrosepticum* SCRI1043 were upregulated in potato tubers, similar to the type VI secretion system genes (III). These findings raised the idea that the *flp/tad* genes and the T6SS could be similarly regulated, at least in part, and that the role of the Flp/Tad pilus in virulence should be assessed. In subsequent studies, it was observed that the *flp/tad* cluster in *Pectobacterium* is highly similar to the previously characterized *flp/tad* cluster in *A. actinomycetemcomitans* and differs slightly from the clusters in *R. solanacearum* and *Pseudomonas* (Wairuri *et al*., 2012; IV). This indicates that the pilus may have divergent properties or structures in different species, which is also supported by the differing structure of the pilus in the strains in which it has been visualized (O’Connell Motherway *et al*., 2011; Tomich *et al*., 2007). The pilus is often a bundle-like structure localized at the pole of a bacterial cell (Tomich *et al*., 2007). The *flp/tad* gene cluster in *Pectobacterium* also contains a novel TCS and mutagenesis of the response regulator in this system had a major effect on transcription of the *flp/tad* genes, indicating that the novel TCS is the regulator of Flp/Tad pilus alone or in collaboration with other regulators (IV). The regulation of the Flp/Tad pilus has been previously investigated only at the transcriptional level, and species-specific features of this regulation were found (Bernard *et al*., 2009; Kram *et al*., 2008; Perez *et al*., 2006; Schilling *et al*., 2010). The role of the sigma 54-dependent transcriptional activator VasH, which is expressed from the T6SS core cluster and regulates the *hcp* and *vgrG* genes (Bernard *et al*., 2009), was also inspected with regard to the regulation of the *flp/tad* gene cluster. However, no regulation of *flp/tad* genes by VasH was demonstrated despite promising microarray results (IV). Previous global gene expression studies in *Pectobacterium* indicated that the *flp/tad* gene cluster in *Pectobacterium* is not regulated by the virulence repressor RsmA (Kõiv *et al*., 2013) or quorum sensing (Expl, quorum molecule synthetase) (Liu *et al*., 2008). It is possible that the TCS flanking the *flp/tad* gene cluster in *Pectobacterium* encodes a novel regulator that solely regulates the Flp/Tad pilus in response to signals that indicate the presence of the host plant (IV). Mutagenesis of the partial *flp/tad* gene cluster and the response regulator of the novel Flp/Tad TCS of *P. atrosepticum* SCRI1043 delayed maceration of potato tubers in comparison to the wild-type strain. A similar phenomenon was repeatable with an Flp/Fap pilin component mutant of *P.*
**wasabiae** SCC3193, indicating that the Flp/Tad pilus and flanking TCS compose a novel virulence determinant in soft-rot enterobacteria (IV).

The function of the Flp/Tad pilus in *Pectobacterium* is unknown. Biofilm assays did not provide information about the function of this pilus (IV). The case of the potato pathogen *R. solanacearum*, in which a TadA2 mutation increased biofilm production (Wairuri *et al*., 2012), suggested that the Flp/Tad pilus may be related to the reduced aggregation of bacterial cells and the promotion of dissemination. Alternatively, this example may suggest that the presence of Flp/Tad pili disturb attachment of type IVa pili and subsequently that the mutagenesis of TadA2 enhances type IVa pili-mediated biofilm formation. In the case of *R. solanacearum*, it was thought that the Flp/Tad pilus was also utilized for the secretion of virulence factors (Wairuri *et al*., 2012), but no evidence was presented for such phenomena. In plant pathogenic bacteria and possibly other bacterial groups, the biogenesis of the pilus could be strictly regulated. In addition to performing investigations at the transcriptional level, other aspects of pilus formation should be studied, as the pilus may be functional only in specific conditions, adding special challenges for the study of the Flp/Tad pilus. In the future, it would be interesting to continue to study bacterial biofilms in plant tissue and the role of the Flp/Tad pilus in this process.

### 4.2.3 SirB is a novel virulence determinant of unknown function

In a genomics-based investigation of novel virulence-related genes in *P. wasabiae* SCC3193, genomic information was assessed based on several parameters to identify putative virulence genes for empirical studies (II). One of the parameters for gene selection was that the gene must have been reported to be related to virulence or host-colonization in another organism or organisms, although the gene itself was not particularly well-studied. Based on this parameter, the SirB locus was identified. SirB is highly conserved within Enterobacteriaceae, but its function is not known (II). In *Salmonella*, the SirB locus is related to virulence and invasion of host cells, but is not crucial, and its exact function is not known (Rakeman *et al*., 1999; Strohmaier *et al*., 1995). However, it is thought that SirB regulates virulence, although no direct evidence has been presented thus far. When SirB is overexpressed in *Salmonella*, it suppresses the phenotype of a SirA mutant, which is a response regulator of a TCS regulating T3SS and other genes in *Salmonella* pathogenicity island 1 (SPI1). This affects virulence and invasion of host cells and is needed for expression of the transcriptional regulator SirC within SPI1 (Johnston *et al*., 1996; Rakeman *et al*., 1999). The SirB locus is composed of two ORFs. One of these ORFs (*sirB2*) encodes a predicted membrane-bound protein, and the other ORF (*sirB1*) encodes a protein that does not contain any recognizable domains that could be soluble, making it the best candidate to be a novel regulator (II). Interestingly, the genomic areas surrounding
the SirB locus are highly conserved and well characterized in *E. coli* and are an elementary part of basic metabolism affecting protein synthesis, LPS synthesis and 3-deoxy-D-[manno]-octulosonic acid metabolism (Nakahigashi *et al.*, 2002; Ryden *et al.*, 1986; Strohmaier *et al.*, 1995). In this study, mutagenesis of the SirB locus of *P. wasabiae* SCC3193 decreased the virulence of bacteria in potato tubers to a large extent (II). To my knowledge, this is the first time that a significant reduction in virulence was reported for a SirB mutant strain of any organism, despite the fact that the SirB locus is highly conserved. In SCC3193, the mutagenesis of SirB was complemented in trans with the addition of the full locus or the ORF encoding the putative soluble protein (*sirB1*), indicating that SirB1 could represent a novel virulence determinant in soft-rot enterobacteria (II). The major reduction of virulence of *P. wasabiae* SCC3193 lacking the SirB locus may indicate that SirB participates in basic metabolism, similar to the surrounding genomic neighborhood in *E. coli*. Alternatively, this virulence reduction may suggest that SirB is a novel major regulator of virulence determinants such as PCWDE or that it functions as a virulence factor *per se*. Despite efforts during this study, it was not possible to identify an effect of SirB on the basic metabolism or major virulence determinants of SCC3193. The SirB mutant and wild-type strains grew similarly in minimal and rich media, and both similarly produced extracellular enzymes (II). SirB could play a specific role during growth *in planta*; thus, the *in vitro* experiments have not provided answers. The investigation of the role of SirB is ongoing, and the secret of SirB will hopefully be revealed in the near future.

### 4.2.4 The HopL1-like protein is present in *Pectobacterium wasabiae* lacking a type III secretion system

In this study, two putative T3SS effectors encoding genes that are similar to SrfC in *Salmonella* and HopL1 (HopPtoL) encoding genes in *Pseudomonas* were characterized in *P. wasabiae* SCC3193 (II). However, only one of these effectors encoding gene (W5S_0495) is unique to *P. wasabiae* strains among soft-rot enterobacteria; the other (W5S_2361) is a widely distributed ORF (II). Interestingly, the ORF (W5S_0495) with the closest sequence similarity to the HopL1 effector (query coverage 99%, identity 42-43%) is present in a cluster encoding a predicted lipoprotein transportation system with sequence similarity to ABC transporters, present in only a few bacterial species whose genomes have been sequenced (II). The HopL1-like protein-carrying gene cluster present in *P. wasabiae* is also present in *P. syringae*, in which the HopL1 protein was shown to be a T3SS secreted putative effector (Petnicki-Ocwieja *et al.*, 2002). All other strains carrying the hopL1-containing gene cluster identified in this study lack a T3SS, indicating that the putative lipoprotein transportation system or other machinery exports HopL1 to the
extracellular milieu if the hopL1 gene is functional (II). The gene cluster encoding the putative lipoprotein transportation system and the HopL1 effector is located in a predicted horizontally acquired island (GI_7) in P. wasabiae SCC3193 and composes the majority of the island (II). Additional genes in GI_7 may also be related to the cell wall, surface structures or secretion (II). Mutagenesis of the hopL1-like gene-containing GI_7 delayed P. wasabiae SCC3193 (Vic2 mutant) disease development on potato tuber slices, suggesting that HopL1 and the surrounding lipoprotein transportation system could play roles in the virulence of P. wasabiae (II). The diversity of the strains carrying hopL1 gene clusters similar to the plant pathogenic P. wasabiae and P. syringae strains is notable and includes plant-, animal- and insect-associated bacteria. Enterobacter sp. 638 is an endophyte of poplar (Lodewyckx et al., 2002), Xenorhabdus bovienii is a mutualist of (entomopathogenic) nematodes and a pathogen of insects (Herbert & Goodrich-Blair, 2007; Richards & Goodrich-Blair, 2009) and Azotobacter vinelandii is a soil bacterium with agricultural importance. The latter is an N-fixing and plant hormone-producing organism that may be associated with plant roots (Kennedy & Toukdarian, 1987). Furthermore, P. multocida subsp. multocida is part of the normal oral flora of animals and is commonly transmitted to humans or other animals by bites, causing disease; in addition, other disease forms also appear (Guet-Revillet et al., 2013; Wilkie et al., 2012). Pseudomonas putida is a versatile species with several biochemical properties that could be utilized in biotechnology. Some of the strains are plant growth-promoting rhizospheric and endophytic bacteria (Wu et al., 2011), whereas Haemophilus parainfluenzae is also part of normal human flora, though in rare cases, it can be a pathogen, causing infective endocarditis (heart infection) (Das et al., 1997). All of these bacterial species that have similar HopL1 gene clusters described in this study have in common that they all can interact with eukaryotic hosts such as plants, animals or nematodes without disease development. Thus, perhaps the HopL1 cluster is beneficial for colonization of a host instead of disease development. Among many plant- and animal-associated bacteria, the T3SS is utilized to suppress the first line of defense that is triggered by microbial- or pathogen-associated molecular patterns (MAMP/PAMP), such as flagella, or to otherwise modify host physiology to enable bacterial colonization (Jones & Dangl, 2006; Ronald & Beutler, 2010). Perhaps the delayed disease development of the Vic2 mutant in our study (II) is a consequence of delayed adaptation to the host environment in comparison to the wild-type strain; however, due to this strain’s massive production of PCWDE, disease was established. GI_7 also contained other putative secreted proteins that may function as effectors, but no function was predicted for these proteins in this study. More studies are needed to show that HopL1 is actually a secreted protein in T3SS-deficient strains and that the adjacent predicted transportation system is responsible for its secretion or to determine which gene or genes are responsible for host colonization or virulence.
5 CONCLUDING REMARKS

This study clarified the taxonomy of the model strain *Pectobacterium wasabiae* SCC3193 using genomic information and identified novel virulence-related genes in soft-rot enterobacteria. These genes were specifically examined in *P. wasabiae*, which is considered an emerging pathogen worldwide, and in *P. atrosepticum*, which is a devastating blackleg pathogen. Interestingly, *P. wasabiae* strains that were isolated from potato share higher sequence similarity and gene composition than the type strain isolated from Japanese horseradish, indicating that there may be two subspecies of *P. wasabiae* that colonize these different plants. However, more studies are needed to define the taxonomic relationship of these two clades. The virulence and fitness in planta are actually very complex in soft-rot enterobacteria, and several traits could be utilized to overcome MAMP-triggered immunity in planta and to coordinate the virulent stage to adjust to the host physiology. In this study, a few novel virulence determinants were characterized that may be needed for virulence, specifically at the early stage of plant colonization. Such traits were the type VI secretion system, the virulence cluster 2 carrying the type III secretion system effector HopL1-like protein and a functionally unknown virulence determinant called SirB. Other novel traits characterized in this study are the Flp/Tad pilus and its regulator, which may be mainly necessary at the maceration stage. These novel findings could explain, at least in part, how soft-rot enterobacteria colonize the host plant and enhance survival during the maceration stage. However, further studies are necessary to confirm the hypotheses regarding these targets and to understand the roles of these novel virulence determinants.
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