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LIIS ANDRESEN

Regulation of virulence in plant-pathogenic pectobacteria

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Regulation of virulence in plant-pathogenic pectobacteria

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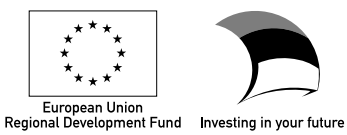

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LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following original publications which are referred to by Roman numerals in the text:

I. **Andresen, L.**, Kõiv, V., Alamäe, T., and Mäe, A. (2007). The Rcs phosphorelay modulates the expression of plant cell wall degrading enzymes and virulence in *Pectobacterium carotovorum* ssp. *carotovorum*. *FEMS Microbiology Letters*, 273:229–238.

II. **Andresen, L.**, Sala, E., Kõiv, V., and Mäe, A. (2010). A role for the Rcs phosphorelay in regulating expression of plant cell wall degrading enzymes in *Pectobacterium carotovorum* subsp. *carotovorum*. *Microbiology*, 156:1323–1334.

III. Kõiv, V., **Andresen, L.**, Broberg, M., Frolova, J., Palva, T., Pirhonen, M., Tenson, T., and Mäe, A. Lack of RsmA-mediated control results in constant hypervirulence, cell elongation and hyperflagellation in *Pectobacterium wasabiae*. Submitted for publication in *PLoS ONE*.

IV. Kõiv, V., **Andresen, L.**, and Mäe, A. (2010). AepA of *Pectobacterium* is not involved in the regulation of extracellular plant cell wall degrading enzymes production. *Molecular Genetics and Genomics*, 283:541–549.

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My contribution to the journal articles referred to in this dissertation is the following:

Ref. I Performed the experiments, contributed to the writing of the manuscript

Ref. II Designed and performed the experiments, analysed the data, participated in writing the manuscript

Ref. III Performed the microarray analysis, participated in writing the manuscript

Ref. IV Performed the virulence tests

ABBREVIATIONS

COG	clusters of orthologous groups; phylogenetic classification system for proteins encoded in complete genomes
HBB	hook basal body; membrane-integrated protein complex forming a basal structure for the flagellar filament
HSL	N-acyl homoserine lactone; quorum sensing signaling molecule
OCS	one component system; small molecule-binding regulators; the simplest class of signal transduction systems
P. atrosepticum	*Pectobacterium atrosepticum*
P. c. ssp. *carotovorum*	*Pectobacterium carotovorum* subspecies *carotovorum*
P. wasabiae	*Pectobacterium wasabiae*
Rcs	signalling system components first identified as regulators of capsule synthesis in *Escherichia coli*
Rsm	global regulatory system homologues of which are widely represented in bacteria; name comes from its identification in *Pseudomonas* as regulator of secondary metabolites; its homologue in *Escherichia coli* is named Csr system derived from carbon storage regulator
TCS	two component system; signaling system consisting of membrane bound sensor kinase and cytoplasmic response regulator

INTRODUCTION

A small blotch on my apple does not disturb me much. However, when this small blemish develops into a fast-growing slimy rot lesion I am forced to discard the apple. One apple is not a big economic loss for me, but an upsurge of these disease symptoms in the fruit or vegetable storages of farmers or companies leads to more serious expense.

Various diseases damage the plant species we use for food, causing economic loss in developed countries and undernutrition in developing countries. Knowing the devastator is the key to precluding the damage, so plant diseases have been under continuous investigation. Although plants can be harmed by abiotic factors, insects and animals, many of their diseases are caused by microorganisms: viruses, fungi, and bacteria. The subject of this thesis is the *Pectobacterium* genus of plant-pathogenic bacteria. Bacteria belonging to this group attack a wide range of crops globally. At present there are no chemical control measures to fight the disease and the control of pectobacterial infection mainly depends on avoidance of *Pectobacterium* contamination on plants and adjusting storage conditions so they do not favour development of the disease. The reason for this is lack of knowledge that could enable us to restrict the onset of disease development specifically. Therefore we have dedicated our research to investigating the regulation of virulence of in these pathogens in order to provide a knowledge base from which it should be possible to work towards more specific, more effective and safer ways to control disease. With more effective disease control measures we can ensure the provision of more food with better quality to the continually growing human population.

REVIEW OF THE LITERATURE

Plant disease characterized by fast-developing wet rotting of the plant stems, tubers or fruits is named soft rot disease (Agrios, 1997). Nearly all fleshy fruits, vegetables and even ornamentals can be subject to soft rot, making this disease more significant. Although bacterial soft rot occurs on a wide range of crops, it is mostly known as one of the most severe post-harvest diseases of potatoes. Potatoes provide 2% of the world's plant-derived food supply (energy per capita per day), placing them 4th in importance after rice, wheat and maize (FAOSTAT, 2007). In Europe, where rice and maize cannot be grown for human food, the potato has an even more substantial role, providing almost 5% of the food supply, which makes it the 2nd crop after wheat. This makes the potato the most important food plant attacked by soft rot bacteria; none of the potato varieties grown today are immune to them.

The most common bacteria causing soft rot symptoms on potatoes belong to the genus *Pectobacterium* (formely classified as subspecies of *Erwinia carotovora*). *Pectobacterium* species are highly motile rod-shaped Gram-negative bacteria that can grow between the temperatures of 0 and 32°C and are therefore found worldwide (Agrios, 1997; Pérombelon *et al.*, 1987). Being a common soil resident, *Pectobacterium* can reside on potato plants in a latent form without causing disease. When environmental factors, such as moisture and temperature, favour disease development, *Pectobacterium* can attack plants in the field. More often, the dormant bacteria are transferred with the crop to storage and the most severe disease symptoms appear in tuber piles. Bacteria enter the potato tubers via natural openings and wounds caused during transit or handling. In the plant apoplast, *Pectobacterium* starts to produce enzymes that catalyze the breakdown of plant cell walls, providing the food necessary for further multiplication. During the infection process, bacteria continue to move and to multiply in intercellular spaces while their enzymes advance ahead of them and prepare the tissues for invasion. As the bacterial population grows, more and more plant cell wall degradation products accumulate in the apoplasmic fluid causing the plant cells to lose water by osmosis. Thereupon, the host cells collapse and die. If it is not properly stored, the whole tuber can be converted within five days to a wet mushy mass consisting of innumerable bacteria swimming about in the liquefied substance. Although the bacteria cannot pass the tuber epidermis, cracks are usually present through which the decayed mass can ooze out and infect neighbouring tubers. Therefore, when potatoes are stored to be used as food, having a fast-spreading *Pectobacterium* infection in the tuber pile means a big if not a total loss of product. If the stored tubers are used as seed, latently infected tubers carry the bacteria into the soil where they can spread to progeny tubers that are potential new victims of the disease.

1. Factors determining the virulence of pectobacteria

The virulence of *Pectobacterium* species is strictly dependent on the massive production and secretion of plant cell wall-degrading enzymes, the action of which is the direct cause of disease symptoms (Pérombelon, 2002). As their name indicates, pectobacteria can catalyze the breakdown of the main plant cell wall component, pectin. This polysaccharide is hydrolyzed by a set of extracellular enzymes produced by the bacterium. A polygalacturonase encoded by the gene *pehA* randomly cleaves D-galacturonan, which is the main component of pectin (Saarilahti *et al.*, 1990). Pectin methyl esterases prepare the pectin for cleavage by several pectate lyases, which act on the de-esterified pectin and strongly contribute to the virulence of the bacterium (Barras *et al.*, 1994; Heikinheimo *et al.*, 1995; Toth *et al.*, 2003). Why the bacterium produces two types of enzymes both capable of pectin degradation is not clear, but it has been suggested that polygalacturonase is needed in the early stages of infection whereas different pectate lyase isoforms become more important after the infection is established (Flego *et al.*, 1997; Pagel & Heitefuss, 1990; Saarilahti *et al.*, 1992). In addition to pectin, plant cell walls contain cellulose and various proteins. At least one cellulase produced by pectobacteria causes virulence symptoms in host plants (Mäe *et al.*, 1995). Similarly, an extracellular protease, PrtW, has been shown to be important for the virulence of the pathogen (Marits *et al.*, 1999). By catalyzing non-specific cleavage of proteins, PrtW is thought to degrade protein components of the plant cell wall. It has also been speculated that it is involved in suppressing plant defences by degrading plant defence system proteins. Irrespective of the actual substrate for each individual enzyme, they all act in concert to cause rotting of the plant.

Although destructive to plants, plant cell wall-degrading enzymes are important for the bacterium. They furnish the bacterium with nutrients essential for its reproduction during the course of the infection. Pectinases and cellulase are important for obtaining carbohydrates, whereas the protease has the potential to provide the bacterium with amino acids. Thus, *Pectobacterium* species act as plant parasites, using the plant for their vital functions and at the same time causing the host organism a loss of nutrients, destruction of tissues, and pollution with metabolic residues.

Motility has also been shown to be important for pectobacteria to establish themselves within the host. Hossain and coworkers (2005) described the ability of two different non-motile mutants (non-flagellated, and flagellated but paralyzed) to cause virulence symptoms in Chinese cabbage. They found that although the non-motile mutants produced the same amount of plant cell wall-degrading enzymes as the parent strain, their ability to elicit soft-rot symptoms was reduced. As expected, the non-motile mutants did not spread away from the point of inoculation to uninfected tissues as well as the wild type bacterium, indicating that motility is important for the development of symptoms because it enables the bacterium to infect more distant tissues. In addition, flagellum production has been shown to be essential for biofilm formation by *Pectobacterium* (Hossain &

Tsuyumu, 2006). Bacteria in biofilms are more resistant to adverse environmental conditions such as desiccation and extreme temperature (Costerton *et al.*, 1994; Dewanti & Wong, 1995; Zottola & Sasahara, 1994) and are thought to be protected from host defence responses (Kharazmi, 1991; Leid *et al.*, 2002; Walker *et al.*, 2004). Thus, flagellum synthesis might also contribute to the virulence of *Pectobacterium* because it is a prerequisite for biofilm development.

In addition to the aforementioned virulence factors, pathogenic bacteria are known to produce virulence factors with the specific aim of evading or suppressing the host's innate immunity. Although different strategies are used by different plant-associated bacteria, the effector proteins delivered directly into the host cytoplasm by the secretion systems play key roles in altering host responses (Rêgo *et al.*, 2010). There are three different secretory pathways that allow bacterial effectors to be inserted directly into the host cells: type III, type IV, and type VI secretion systems (named according to their order of discovery). Although there is only limited information about the effectors of these secretion systems and their role in the virulence process of pectobacteria, all these pathways are represented in the genus *Pectobacterium*. The genome of *Pectobacterium atrosepticum* strain SCRI1043 encodes all of the named secretion systems and they all affect the virulence phenotype of the bacterium in the host plant (Bell *et al.*, 2004; Holeva *et al.*, 2004; Liu *et al.*, 2008). Interestingly, several *Pectobacterium* species, including the *Pectobacterium wasabiae* strain SCC3193 (formerly identified as *Pectobacterium carotovorum* subsp. *carotovorum*) used by our research group, do not encode functional type III (encoded by the *hrp* genes) or IV secretion systems (Glasner *et al.*, 2008; Kim *et al.*, 2009; Koskinen *et al.,* in press). Thus, these species use different mechanisms to transport effector molecules into the host cells. One such alternative mechanism could be the type VI system previously shown to inject *Vibrio cholerae* effector molecules into mammalian cells (Pukatzki *et al.*, 2007). The genes for the type VI secretion system in *Pectobacterium* are plant-induced, and inactivation of this secretion yields a less virulent pathogen (Liu *et al.*, 2008; Mattinen *et al.*, 2008). It is therefore possible that this secretion system is important for inhibiting the host's counterattack against the pathogen. Alternatively, type VI has been shown to be important in *Pseudomonas aeruginosa* and *Vibrio cholerae* for out-competing other Gram-negative bacteria (MacIntyre *et al.*, 2010; Russell *et al.*, 2011). Thus, type VI-dependent secretion could have a role for *Pectobacterium* in establishing itself in bacterial communities, instead of or in addition to its possible role in suppressing the plant defence response.

The purpose of virulence factors in any pathogen is to enable it to survive and reproduce in the host. Thus, the highest levels of virulence factors are produced by plant-pathogenic bacteria in the host plant. Usually, different factors are needed during different stages of infection: during the colonization phase the evasion and suppression of plant defences is most important, whereas after the infection is established, feeding and reproduction become overriding. Under unfavourable growth conditions or outside the host, bacteria repress the uneconomical production of virulence proteins. Instead, they up-regulate genes

important for survival in the stressing condition(s) they confront. In order to control the production of virulence factors according to necessity, a complex regulatory network has evolved in *Pectobacterium* (described under *Regulation of virulence in Pectobacterium*). Regulators in this network are affected by environmental signals and ensure that the production of virulence factors is correlated with the environment. Before discussing the complex regulation of virulence factors, I will give an overview of the way bacteria sense the environment by describing their main tools for signal transduction.

2. Sensing the surroundings

The mechanisms for sensing environmental or intracellular conditions are similar in all bacterial species. They involve proteins with domains capable of sensing the surroundings and domains with regulatory function, together forming bacterial signal transduction systems (Stock *et al.*, 2000). As bacteria reside in extremely diverse habitats, different signal transduction systems are needed to convert a broad spectrum of environmental stimuli to cellular responses to ensure adaptation to each particular environment. While different environments and signals evoke different responses from the cell, multiple distinct signalling systems have evolved in bacteria. The specificity of each system is determined by the input domain, which is able to sense only specific signals, and by the output domain, which affects certain targets.

For perfect signal transduction, the input and output have to be properly linked ensuring a precise and isolated response within the cell. Input and output domains are connected by molecular signalling systems ranging from very simple transcriptional regulators to multi-component, multi-pathway signalling cascades. The following sections introduce bacterial signalling systems from the simplest to the more complicated.

2.1. The simplest form of signal transduction

The simplest and evolutionarily the oldest way to connect the input and output domains is by fusing them into one protein. Signalling proteins containing an N-terminal input domain and a C-terminal output domain are called one-component systems (OCS), ligand-binding regulators or stand-alone regulators (Ulrich *et al.*, 2005). The prototypic OCS has an input domain that is able to bind a small cytosolic molecule. Through that binding the signal is sensed and the conformation of the protein changes. Consequently, the affinity of the C-terminal DNA-binding domain for the target gene promotor changes and it acts as a positive or negative regulator of target gene expression. For example, the DNA binding activity of the KdgR repressor is negatively modulated by binding to pectin degradation products (Nasser *et al.*, 1992). Other well-known examples of OCSs found in many Gram-negative bacteria, including pectobacteria, are quorum sensing regulator proteins, the activities of which depend on direct binding to different acyl-homoserine lactone molecules (Whitehead *et al.*, 2001).

The limitation of OCSs is that they are able to sense and respond to signals present in or reaching the cytosol to adjust mainly to conditions inside the cell. Although it is indisputably important to monitor and regulate intracellular events, it is also important for bacteria to translate and respond to signals from the surroundings. This cannot be done if the input domain is cytosolic and the signal cannot pass through the bacterial membranes. To overcome this problem, Nature has dislocated the input and output domains into different protein molecules capable of information exchange mainly via phosphorylation events.

2.2. Signal transduction via phosphotransfer

The activation of proteins through phosphorylation has been known since the 1960s, when Fischer and Krebs demonstrated that the activity of glycogen phosphorylase depends on its state of phosphorylation (Fischer & Krebs, 1966). Since then, reversible protein phosphorylation has been shown to control a wide array of metabolic processes. In addition, it has been found that sequential protein phosphorylation is a common mechanism for transferring information in prokaryotes as well as eukaryotes.

In bacteria, the protein modules employed by phosphotransfer-based signal transduction systems are basically the same irrespective of the particular system: (i) The histidine kinase domain is capable of ATP-dependent autophosphorylation at a conserved histidine residue in response to conformational changes induced in the input domain by the signal molecule; (ii) the receiver domain, usually linked with the output domain, acquires the phosphate from the phosphorylated histidine kinase domain and transfers it to its conserved aspartate residue; (iii) some signal transduction systems use histidine-containing phosphotransfer domains that mediate phosphate group transfer between receiver domains. The phosphotransfer systems used for signalling in bacteria are built up from these three conserved elements, but their number and organization into proteins can differ from one example to another, forming signalling cascades comprising two to several proteins (Fig. 1).

2.2.1. Two-component systems

The most abundant phosphotransfer-based signal transduction system consists of two proteins: the sensor and the response regulator (Stock *et al.*, 2000; West & Stock, 2001). The membrane-bound sensor protein contains the input domain, which is linked with the histidine kinase domain. The response regulator contains the receiver and the output domain. Upon signal recognition, the sensor protein dimerizes and then the histidine kinase domain undergoes ATP-dependent autophosphorylation. Subsequently, the phosphate group can be acquired from the phosphorylated sensor protein by the N-terminal receiver domain of the response regulator. Once it is phosphorylated at a conserved aspartate residue, the response regulator manifests its regulatory activity. The C-terminal output domain can now

enact the outcome of most signal transduction systems: regulation of gene expression. When environmental conditions change and the signal vanishes, signal transduction must be switched off. This is achieved by dephosphorylation of the response regulator (Stock & Da Re, 2000). Most response regulators can autocatalyze the removal of the phosphate group, ensuring that regulation occurs only when the signal is sensed, *i.e.* when the sensor is phosphorylated. The dephosphorylation can be accelerated by unphosphorylated cognate sensor kinases or distinct bacterial phosphatases, enhancing the fidelity of the system (Blat & Eisenbach, 1994; Tzeng *et al.*, 1998).

One of the most extensively studied two-component systems in *Escherichia coli*, but also present in various other bacterial species including pectobacteria, is the EnvZ-OmpR system. The activity of this system depends on the phosphorylated OmpR response regulator, the level of which is gradually modified by the EnvZ sensor kinase (Comeau *et al.*, 1985). Phosphotransfer in the direction of phosphorylated OmpR is induced in high osmolarity environments, *e.g.* in the mammalian gut (Cai & Inouye, 2002). Upon activation, OmpR acts as a transcriptional regulator of outer membrane porin genes, and through this gene regulation it changes the permeability of the membrane. Interestingly, it has been demonstrated that OmpR can also act as a transcriptional regulator irrespective of its state of phosphorylation by binding to high-affinity sites in some target gene promoters (Bergstrom *et al.*, 1998; Goh *et al.*, 2004), demonstrating that signal transduction components can be involved in gene regulation even in the absence of an activating signal.

Although most phosphotransfer-based signalling systems in bacteria have the structure of a classical two-component system, two-component system variants exist in which additional phosphotransfer modules are involved.

2.2.2. Phosphorelays

The terms ´two-component system` and `phosphorelay` have been used to categorize phosphorylation-dependent signalling systems in bacteria. The line between these categories is most often drawn according to the number of protein components involved in phosphotransfer. However, I find it more appropriate to classify these signal transduction systems on the basis of the number of phosphorylation/dephosphorylation events that take place during information transfer. Thus, I use the term `two-component system` in this thesis only for a signal transduction in which just one phosphotransfer event, in addition to autophosphorylation of the sensor protein, is needed to deliver the signal (as described in the preceding section). Signal transduction systems that involve more than one phosphotransfer event between the histidine- and aspartate-containing domains before the phosphate group arrives at the response regulator are called ´phosphorelays´ in this text (Fig. 1). The reason for this classification is that some phosphorelays also comprise two protein components, but act by just the same mechanism as phosphorelays that contain the same phosphotransfer modules divided among more than two proteins.

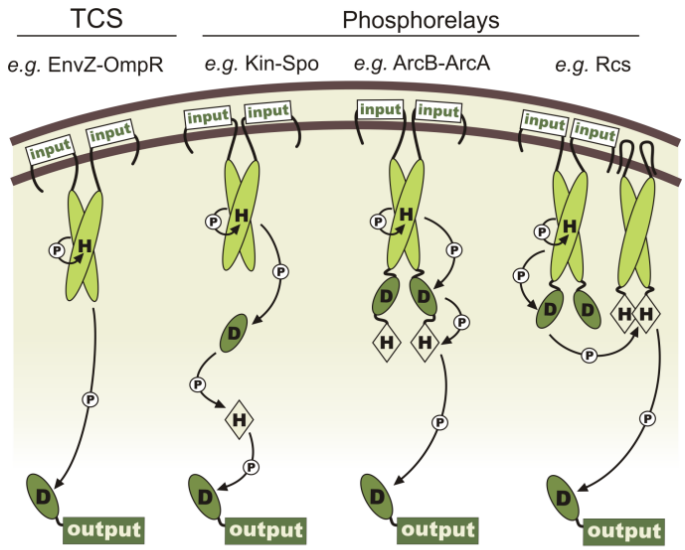

Figure 1. Phosphotransfer-based signalling systems in bacteria. The system-specific signal is sensed by the input domain of the membrane-bound sensor kinase exposed to the periplasmatic space. Signal recognition induces autophosphorylation of the histidine residue (H) in the kinase domain of the sensor (light green). Subsequently, the phosphate can be acquired by the receiver domains (dark green, D = aspartate). The receiver domains can reside in response regulators, as in two-component systems (TCS) where the phosphorylation of the cytoplasmic response regulator designates the end of the signal transduction. However, in phosphorelays, the receiver domains can be incorporated in a separate protein (*e.g.* Kin-Spo) or be a part of a sensor protein as in the Arc and Rcs phosphorelays. As phosphate transfer in bacteria occurs strictly between H and D residues, an additional protein domain, the histidine-containing phosphotransfer domain (rhomb), is recruited for signal transduction by the relays. The phosphorylation of the response regulator changes the activity of its output domain, which determines the cellular response to the signal.

Phosphorelays employ the same protein domains described for two-component systems (input, histidine kinase, receiver, and output domains), but also utilize a histidine-containing phosphotransfer domain that enables phosphate to be transferred from one receiver domain to another (Fig. 1). Because they have more modules involved in phosphotransfer than classical two-component systems, phosphorelays do not necessarily consist of two signalling proteins. Instead, they can comprise up to four proteins involved in sequential phosphotransfer, as in the Kin-Spo system in *Bacillus subtilis* (Piggot & Hilbert, 2004; Fig. 1).

Interestingly, some phosphorelays can also act as two-component systems. For instance, in the ArcB-ArcA signal transduction system, important in regulating energy metabolism, the phosphate group travels different paths within the system depending on the availability of anaerobic electron acceptors. Under most conditions the phosphate is transferred directly from the kinase domain of the sensor to

the receiver domain of the response regulator, skipping the receiver and phosphotransfer domains of ArcB (Georgellis *et al.*, 1997). However, in response to additional anaerobic electron acceptors, signalling through the Arc system becomes dependent on the phosphotransfer domain of ArcB, indicating that all the signalling modules are used in these conditions (Matsubara & Mizuno, 2000). Therefore the presence of additional phosphotransfer modules makes the phosphorelays more flexible than the two-component systems.

Another way in which the extra His-Asp phosphotransfer components in phosphorelays are important, compared to two-component systems, is that they can serve as additional regulatory checkpoints in the signalling pathway. For example, a signalling cascade can be blocked by or relieved from cellular phosphatases that dephosphorylate the intermediate components of phosphorelays (Missiakas & Raina, 1997). In addition, the signalling module itself can act as a regulator of signal transduction. This has been demonstrated in the VirA-VirG system in *Agrobacterium tumefaciens*, where the receiver domain of the VirA sensor functions as an intramolecular repressor of the kinase activity under certain conditions, blocking signalling to the VirG response regulator (Chang *et al.*, 1996).

One signal transduction system, found exclusively in enterobacteria and explored in the *Results and Discussion* section of this thesis, is the Rcs phosphorelay. The protein components participating in the phosphotransfer events are the membrane-bound histidine kinase RcsC and phosphotransfer protein RcsD (also known as YojN) and the cytoplasmic response regulator RcsB (Fig. 1; Majdalani & Gottesman, 2005). Although the actual signalling molecule that activates the RcsC sensor protein is unknown, several conditions have been shown to induce phosphotransfer within this system (reviewed in Clarke, 2010). For example, it has been demonstrated that molecules that disturb the integrity of the membrane or peptidoglycan activate the Rcs-dependent regulation of target genes (Farris *et al.*, 2010; Laubacher & Ades, 2008). Current knowledge suggests that the phosphorylation of RcsB is dependent on both of the membrane-integrated proteins under these conditions; the phosphate cannot be acquired directly from the sensor kinase RcsC, but must follow the path from RcsC to RcsD and from the latter protein to RcsB. In *E. coli*, phosphoactivated RcsB forms homodimers or heterodimers with auxillary proteins, then binds and regulates target gene promoters (Castanié-Cornet *et al.*, 2010; Nagahama *et al.*, 2006; Venkatesh *et al.*, 2010), though some of the known target genes do not need activated RcsB for their regulation. For example, the RcsB-BglJ dimer in *E. coli* activates *leuO* expression independently of Rcs signalling (Stratmann *et al.*, 2012), indicating that this response regulator can also be active in the absence of an activating signal. In addition to BglJ proteins, RcsB can interact with GadE, TviA, RcsA and possibly other regulators in the cell, indicating that the response to the stimulus sensed depends not just on the signalling activity of the system, but also on the presence of other regulatory proteins (Castanié-Cornet *et al.*, 2010; Nagahama *et al.*, 2006; Virlogeux *et al.*, 1996).

2.3. Signaling systems are webbed into complex regulatory networks

In natural environments, bacteria are exposed to many different signals/stimuli, so several signalling systems are activated simultaneously. The cellular regulatory network integrates these signals into the overall response delivered by the cell. In some cases the different signals induce congruent responses while in others they elicit opposing outcomes. Thus, signal transduction systems, frequently with the help of other regulatory systems, affect each other's activities to ensure that exactly the right systemic response to specific conditions is delivered by the cell. In conditions where it is important to block the transduction of one stimulus when a contrary environmental signal is received by another signalling system, several mechanisms can be used. The most energy-saving mechanism is to inhibit the autophosphorylation of the sensor protein (an example is given in Goodman *et al.*, 2009). Alternatively, signalling can be blocked or attenuated by up-regulating the system-specific phosphatase genes or down-regulating the genes encoding the signalling components of the system targeted. Similarly, under conditions where the simultaneous activation of two signalling systems is needed, one signal-activated system can induce phosphotransfer in the other, for example by blocking the dephosphorylation of the response regulator (Kato *et al.*, 2007) or inhibiting the expression of phosphatase genes or up-regulating the expression of component signalling proteins. In addition, there is constitutive competition of response regulators as well as OCS regulators for target gene promoters, strongly modulating the overall output of the signalling systems.

For example, the PhoQ-PhoP two-component system affects the activity of the PmrB-PmrA system in *Salmonella enterica* (Kato *et al.*, 2007). In environments with low Mg^{2+} concentrations, the PhoQ sensor kinase actively phosphorylates its cognate response regulator PhoP. Phospho-activated PhoP in turn activates the expression of its target genes, including *pmrD*. Increasing levels of PmrD inhibit dephosphorylation of the response regulator of the Pmr system, making this system constitutively active. Although activation of the Pmr system in response to Mg^{2+} concentrations is slower than to its primary signal, Fe^{3+}, the resulting response is stronger and more persistent.

In conclusion, signal transduction and regulation of gene expression are tightly coupled in bacteria, forming a complex signalling-regulation network in which most of the components have the capacity to affect others in order to amplify, modulate, or repress the other's response, ensuring the adaptation of the bacterium to the particular environment. Appropriate physiological adaptations are crucial for pathogens that have to adjust to conditions outside the host as well as to survive attack by the immune system and other conditions encountered within the host organism. Thus, in *Pectobacterium* species, as in other pathogenic bacteria, the expression of many traits, including the synthesis of virulence factors, is tightly controlled by environmental cues.

3. Regulation of virulence in *Pectobacterium*

The environment surrounding the pathogen affects the synthesis of virulence factors both directly and by altering the physiological state of the bacterial cell. When the bacterium resides in the soil, on a plant surface or in an insect vector, it has no need to produce factors for virulence. In contrast, if the bacterium infects a plant and starts to reproduce, virulence factors are essential for acquiring food and establishing the bacterial population within the host. The decision on whether to attack or stay latent depends on signals from the surroundings, which through a complex of signalling-regulatory pathways control the expression of appropriate genes. The following sections review today's knowledge about the regulatory network controlling virulence in plant pathogens known to belong to the genus *Pectobacterium*.

3.1. Repression of virulence genes

High oxygen levels, low humidity, and various other conditions that do not favour the development of disease inhibit the production of virulence factors in *Pectobacterium* species (Pérombelon, 2002), though in most cases the regulators that mediate the cellular response to such conditions are not known. It is possible that the absence of a virulence-inducing signal is sufficient in itself to preclude the synthesis of virulence proteins. However, there are two known OCS regulators that directly repress the transcription of genes encoding plant cell wall-degrading enzymes in *Pectobacterium:* KdgR and LrhA[1] (HexA). KdgR is a repressor of several genes for pectin catabolism in a plant pathogen now excluded from the genus *Pectobacterium* and reclassified as *Dickeya* (formerly *Pectobacterium chrysanthemi*; Samson *et al.,* 2005; Nasser *et al.*, 1994). It blocks the synthesis of proteins required for pectin assimilation (including pectinases) until there is contact with the host plant. LrhA has also been shown to bind pectinase gene promotors and repress their expression, but the conditions under which the repression is relieved remain unclear (Harris *et al.*, 1998). Not only do KdgR and LrhA directly regulate pectinase synthesis, they also regulate virulence genes by affecting the expression of components of the Rsm system (Liu *et al.*, 1999; Mukherjee *et al.*, 2000).

The Rsm system plays a central role in virulence regulation in several plant and animal pathogens (Lucchetti-Miganeh *et al.*, 2008). It is extremely important in pectobacteria because many of the signalling pathways controlling virulence gene expression depend on it (Fig. 2). The acting component of the Rsm system is an RNA-binding repressor protein, RsmA, which destabilizes the mRNAs of virulence genes possibly by blocking their translation (Ma *et al.*, 2001). RsmA is antagonized by the regulatory component of this system, RsmB RNA, an untranslated RNA that can compete for RsmA binding, thus lowering

[1] LrhA is considered an OCS regulator on the basis of its predicted domain structure; it contains both the DNA- and substrate-binding domains described in the conserved domain database (Marchler-Bauer *et al.*, 2011). The ligand for this regulator has yet to be identified.

the levels of free RsmA (Liu *et al.*, 1998). As a post-transcriptional regulator, RsmA has the potential to override the regulatory effects at the level of transcription and to act as an important node in the regulatory network by translating different environmental signals into the production of the requisite amounts of virulence factors.

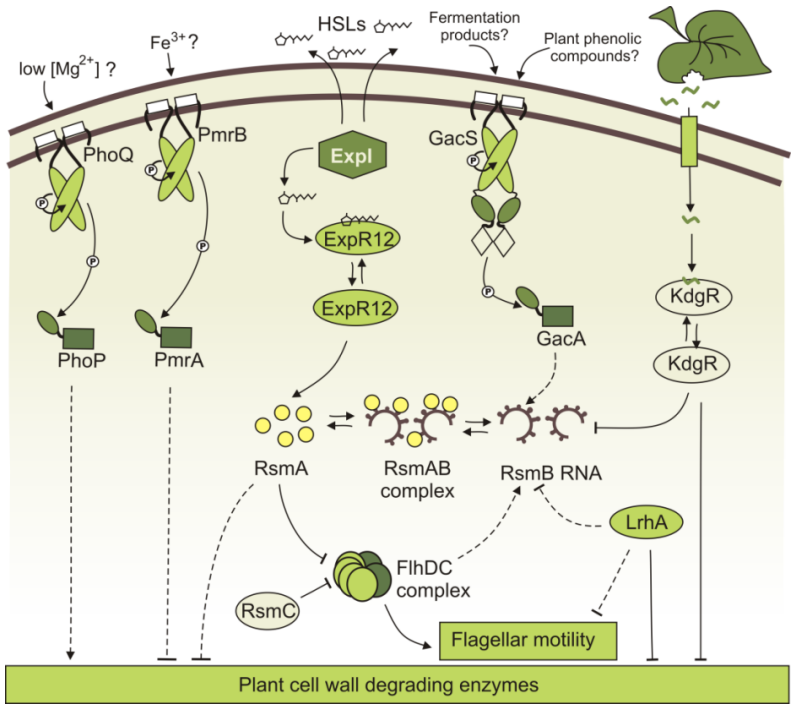

Figure 2. The regulatory network controlling the virulence of pectobacteria. The expression of plant cell wall-degrading enzymes is affected by phosphotransfer-based signalling systems such as PhoQ-PhoP (PehS-PehR), PmrB-PmrA, and GacS-GacA (ExpS-ExpA), which according to studies of homologous systems in other enteric bacteria respond to concentrations of metal ions, plant phenolic compounds or fermentation products. In addition, the synthesis of virulence factors is affected by the quorum-sensing signals acyl homoserine lactones (HSLs), synthesised by the ExpI protein, and pectin degradation products (green waves). The transcriptional regulators mediating the effects of these signals are ExpRs for quorum sensing and KdgR for host plant-dependent control. The transcriptional regulator LrhA (HexA) has also been shown to contribute to virulence control, although the signal controlling its activity is not known. The Rsm system, consisting of an RNA-binding protein RsmA and its antagonist RsmB regulatory RNA, forms a posttranscriptional virulence control mechanism in the centre of the network. The Rsm system is also important in coupling flagellar motility with virulence factor synthesis by affecting the expression of the flagellar master regulator FlhDC. RsmC acts as a protein antagonist of FlhDC, thereby controlling the biosynthesis of flagella and indirectly (through the Rsm system) the production of virulence factors. Barred lines indicate negative regulation whereas arrows indicate positive effects. Dashed lines indicate interactions that are not proven to be direct.

3.2. Activation of virulence genes *in planta*

During the early stages of infection, when the bacterial count in the host is low, virulence factors are not produced and the bacterium remains latent. There are two possible reasons for that. First, there are enough nutrients in the apoplastic fluid to feed the low number of pathogens, so there is no need to produce plant cell wall-degrading enzymes. Second, if plant cell wall-degrading enzymes are not produced, there are no pectin degradation products (oligogalacturonides) that could evoke potentially lethal plant defence responses. The transmission from latent to infectious lifestyle depends strongly on the bacterial population density at the infection site (Liu *et al.*, 1998). To sense population size and regulate virulence factors accordingly, *Pectobacterium* species use a signal transduction system called quorum sensing. The signalling molecules to which the system responds, acyl homoserine lactones (HSLs), are produced by the bacterium throughout proliferation and accumulate in the milieu of the infection site. HSLs are freely-diffusible molecules, so they can enter and exit the bacterial cells causing the homogenisation of signalling molecule concentration (Miller & Bassler, 2001). HSL levels increase synchronously with the bacterial population. When the bacterial count is high enough for attack against the host to start, the high concentration of HSLs is the signal that induces the production of the large amounts of virulence factors required. The regulatory pathway used for HSL-dependent activation of the virulence phenotype involves quorum sensing regulators, ExpRs (Fig. 2). ExpRs act as OCSs, the activity of which depends on direct interaction with HSLs (Monson *et al.*, 2012). High HSL levels cause the removal of ExpR proteins from the promoter of *rsmA*, the gene encoding a global virulence gene repressor (Cui *et al.*, 2005; Monson *et al.*, 2012), causing a decrease in its transcription. This, in turn, results in the enhanced synthesis of virulence factors required for the infectious lifestyle.

Although it is likely to be irrelevant for the bacterium under natural conditions, HSL-dependent quorum sensing signals population size to the bacterium not only *in planta* but also under other conditions that favour bacterial growth (*e.g.* rich medium). The only truly plant-specific signals currently known to activate virulence are the plant oligogalacturonides. Oligogalacturonides are released from the plant cell wall during the course of pectin hydrolysis by microbial polygalacturonases (D'Ovidio *et al.*, 2004). After internalization, the derivatives of oligogalacturonides act as signalling molecules by binding the KdgR repressor and consequently unleashing its target gene promoters. In addition to the transcription of plant cell wall-degrading enzymes, transcription of the regulatory RsmB RNA is activated (Liu *et al.*, 1999). Thus, plant degradation products also enhance the production of virulence factors post-transcriptionally by increasing their stability through lowering the levels of free RsmA in the cell. It is not known at which stage of infection regulation by oligogalacturonides becomes important, but it is likely that the effects of HSLs and oligogalacturonides on virulence factor synthesis are additive, causing a synergistic response that ultimately results in massive production of virulence factors in well-established infection sites.

The quorum sensing signal and oligogalacturonides both use OCS regulators to mediate their effects on virulence. In addition to OCSs, regulation of virulence in pathogenic bacteria frequently involves phosphotransfer-based signalling (Gotoh *et al.*, 2010). That is also the case in *Pectobacterium* species. PhoQ/PhoP (PehS/PehR) and PmrB/PmrA TCSs, which by homology with similar systems in other enterobacteria are likely to respond to concentrations of metal ions, both affect the virulence of the pathogen (Flego *et al.*, 2000; Hyytiäinen *et al.*, 2003; Fig. 2). The GacS/GacA (ExpS/ExpA) phosphorelay has the strongest effect among the known phosphotransfer-based signalling systems. This system was first shown to be involved in virulence regulation of *Pectobacterium* in 1998 when the group of Professor E. T. Palva demonstrated that the *expA* and *expS* mutants of *Pectobacterium wasabiae*[2] showed reduced virulence (Eriksson *et al.*, 1998). The effect of this system on virulence is now known to depend largely on the expression of plant cell wall-degrading enzymes and to be mediated through the Rsm system (Cui *et al.*, 2001; Hyytiäinen *et al.,* 2001); the response regulator GacA is essential for the expression of *rsmB*. Thus, the level of RsmB RNA is lower in *exp* mutants than in the wild type strain, resulting in higher levels of free RsmA in the cell and reduced virulence of the pathogen. The signal activating phosphotransfer from sensor to regulator is not known, but homologous systems in other bacteria have been shown to respond to plant phenolic compounds and fermentation products (Chavez *et al.*, 2010; Yamazaki *et al.*, 2012). If this is also true in pectobacteria, the GacS-GacA system is potentially induced in response to plant defence responses that involve increased production of phenolic compounds toxic to the intruder, and/or during later stages of infection when enough fermentation products have been released by the bacterium in the course of anaerobic feeding.

Unfortunately, none of the direct targets of phosphotransfer-based signalling systems have been identified in *Pectobacterium* species. Therefore the question of whether plant cell wall-degrading enzymes are *bona fide* targets for the systems described, or whether changes in the production of extracellular enzymes accompany their role in physiological adaptation, is still unanswered. Likewise, the exact signals and the importance of these systems in the infection process remain to be discovered.

3.3. Regulation of motility

Swimming motility is thought to be important in the infection process as it helps the bacteria to spread through the host tissues. The liquid environment needed for swimming results from the action of the plant cell wall degrading-enzymes, which causes water to flow from the plant cells into the apoplastic space (Pérombelon, 2002). At the same time there is no advantage for the pathogen in spreading to more distant tissues if it is not producing the enzymes that enable it to feed in the new environment. Therefore the concurrent needs for both exo-

[2] Identified and classified as *Erwinia carotovora* subsp. *carotovora* at this time.

enzymes and flagellar motility probably explains why the expression of these traits is strongly co-regulated in *Pectobacterium* species.

Motility in pectobacteria is mainly regulated by controlling the expression of the master regulator of flagellar genes, FlhDC. The co-regulation of flagellum synthesis with the production of virulence factors is also achieved on this level: the global repressor of virulence genes, RsmA, directly represses the translation of FlhD protein, resulting in lower expression of flagellar genes (Fig. 2; Chatterjee *et al.*, 2010). For that reason, all the signal transduction systems for which the effects on plant cell wall-degrading-enzyme genes are mediated through the Rsm system also have the potential to affect motility. In addition, the function of FlhDC itself determines the amount of plant cell wall-degrading enzymes produced by the pathogen (Cui *et al.*, 2008). Thus, the regulators that affect the expression or function of the FlhDC complex (*e.g.* RsmC) also regulate virulence, forming a regulatory circle in which motility and virulence are strongly interrelated (Chatterjee *et al.*, 2009, 2010; Cui *et al.*, 2008).

AIMS OF THE STUDY

The regulation of virulence in pathogenic bacteria is an interesting subject for many researchers, not only because of the scientist´s main characteristic – fascination with Nature`s creation - but also because knowing the ways in which virulence is controlled provides options for controlling these pathogens in places where conflicts of interests with the human population arise. The mechanisms of regulation of virulence in pectobacteria have been studied for more than 20 years. Nevertheless, there is still a lack of information concerning the role of several already-identified regulators in the disease process. In addition, ubiquitous regulatory pathways and missing connections among them indicate the presence of unidentified virulence regulators. Therefore the general goal of my studies was to identify novel regulators involved in controlling virulence in *Pectobacterium* and to contribute to clarifying the roles of some of the previously-identified regulators.

The *Results and Discussion* section of the current thesis draws on the results obtained from studies conducted with three specific aims. These are, in the context of the publications underpinning this thesis:

- To identify the role of the Rcs phosphorelay in the synthesis of *Pectobacterium* virulence factors (Ref I, Ref II);
- To identify the traits controlled by RsmA protein in *Pectobacterium* (Ref III);
- To define the role of AepA in the virulence of *Pectobacterium* (Ref IV).

THE OBJECT UNDER STUDY

Studies on the virulence of *Pectobacterium* have been conducted on different species and strains in different laboratories, which may have affected the results obtained. The *Pectobacterium* strain studied by our research group was isolated from soil in Finland (Pirhonen *et al.*, 1988). It is highly motile, virulent in potato, and produces large amounts of plant cell wall-degrading enzymes, so it is well suited for studying the virulence of pathogens belonging to the same genus.

Owing to rapid progress in the taxonomy and identification of bacterial species, the object under study has been renamed twice during the course of my scientific career: it was reclassified from *Erwinia carotovora* subsp. *carotovora* to *Pectobacterium carotovorum* subsp. *carotovorum*, and just recently the specific bacterial strain has been identified as *Pectobacterium wasabiae* on the basis of its genome sequence (Hauben *et al.,* 1998; Nykyri *et al.,* in press).

The species *P. wasabiae* was first isolated from diseased Japanese horseradish (Goto & Matsumoto, 1987). It has recently been shown to cause soft rotting of potato in New Zealand (Pitman *et al.*, 2009). The isolation of the same species from such distant locations as Japan, New Zealand and Finland indicates that the bacterium is widespread. Moreover, its occurrence on both horseradish and potato indicates an ability to infect different host plants. Thus, although the economic losses caused by this particular species are not yet known, it potentially causes the soft rotting of various crops found worldwide. In addition, previous studies of *Pectobacterium* have shown that both virulence and its regulatory mechanisms are essentially similar in different species. It might therefore be possible to extrapolate the knowledge acquired from our research to other related species.

RESULTS AND DISCUSSION

I. The role of the Rcs phosphorelay in the virulence of *Pectobacterium wasabiae*

The starting point of my studies on *Pectobacterium* virulence was rather classical: I began by screening a pool of *P. wasabiae* mutants generated by random insertion of mini-Tn5 transposon coding for antibiotic resistance (chloramphenicol, Ref I). The mutants were plated on milk-supplemented plates and selected according to their ability to produce one of the known virulence factors, the extracellular protease. Colonies that showed an increased ability to degrade milk proteins were identified by large haloes on the milk-plates; those that were unable to produce the same enzyme did not form transparent haloes around the colonies. Since in most cases the production of extracellular protease is co-regulated with other virulence factors, these mutants were regarded as strains in which the transposon insertion had disrupted a gene required for the expression of virulence genes and were selected for further studies.

As a result of this search for genes regulating the production of virulence factors in *P. wasabiae*, I isolated transposon-insertion mutants of the genes encoding the Rcs phosphorelay (Ref I). In other enterobacterial species, this signal transduction system consists of three proteins involved in information transfer: the sensor kinase RcsC, the phosphotransfer protein RcsD, and the response regulator RcsB (vide *Review of the Literature*). The isolated *P. wasabiae rcsC*, *D* and *B* mutants produced elevated levels of plant cell wall-degrading enzymes on the indicator plates and in quantitative assays, indicating that this signal transduction system is involved in inhibiting virulence gene expression (Ref I).

The virulence of pectobacteria is normally repressed outside the host organism. However, the Rcs phosphorelay-dependent inhibition of virulence factor synthesis and overall virulence also occurs *in planta*, as the *rcsB*-negative strain was more virulent when tested on potato tubers (Ref I, Fig. 3). Thus the repression of virulence factor synthesis by the Rcs system cannot be removed in response to conditions in the host plant in this case. Why does the Rcs system need to be active *in planta*? My experiments indicate that inactivation of the regulatory counterpart of the Rcs system reduces the fitness of the bacterium (Fig. 3). Therefore, this system is required to cope with the conditions encountered in the tubers. It is not clear whether the higher or earlier production of virulence factors causes a stronger defence response by the plant organ and explains the lower cell count of the *rcsB*-negative strain in the infected tuber. Alternatively, the Rcs phosphorelay might be required to evade plant host responses or sustain wild type level reproduction independently of virulence factor synthesis.

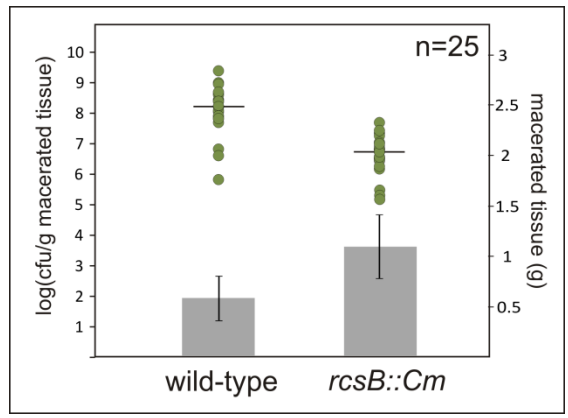

Figure 3. The *rcsB*-negative mutant is more virulent but less viable in potato tubers than the wild type strain. Surface-sterilized potato tubers were infected with approx. 10^6 wild type or *rcsB*-negative strain cells. After 48 hours incubation at 30°C in humid conditions, the macerated tissue was removed from the tuber and weighed (bars), and the number of residing bacteria was determined (dots). Error bars indicate standard deviations. Horizontal lines indicate the median bacterial count in the macerated tissue.

1.1. Target genes of the Rcs phosphorelay important for virulence regulation

In the plant-pathogenic bacterium *Erwinia amylovora*, which causes fire blight on apples and pears, the Rcs signal transduction system directly regulates the expression of virulence genes (Wang *et al.*, 2009; Wehland *et al.*, 1999). To investigate whether the Rcs system in *P. wasabiae* controls the expression of virulence genes directly or feeds its signal into the complex regulatory network controlling the expression of these genes (Fig. 2), I searched for the target genes of the RcsB response regulator using *in silico* target site prediction and by studying RcsB-DNA interactions in the predicted binding sites. In the course of my studies I found a high-affinity binding site for RcsB in front of the *flhDC* operon coding for the master regulator of flagellar genes (Ref II). In addition, I demonstrated that when RcsB is present in the cell, the initiation of transcription from the *flhDC* promoter is repressed. FlhD is essential for virulence in pectobacteria, since the inactivation of *flhD* has been shown to result in a non-virulent strain unable to produce plant cell wall-degrading enzymes (Ref II, Cui *et al.*, 2008; Matsumoto *et al.*, 2003). Thus, the suppression of virulence factors by the Rcs system is likely to be caused by lower levels of *flhDC* mRNA in the presence of RcsB. This is further supported by my results showing that inactivation of *rcsB* has no effect on the synthesis of plant cell wall-degrading enzymes in the absence of FlhD (Ref II).

The effect of *flhD* on the production of plant cell wall-degrading enzymes is indirect and involves modulation of the RsmB RNA levels (Cui *et al.*, 2008, Fig. 2). Thus the negative effect of the Rcs system on the initiation of *rsmB*

transcription, seen in my experiments, was expected (Ref II). Interestingly, my *in silico* analysis identified an RcsB binding site in front of the *rsmB* gene. *In vitro* binding experiments demonstrated that although unphosphorylated RcsB could not bind to this site, the phosphorylated or constitutively active form bound the predicted site as well as an adjacent site in the *rsmB* regulatory region (Ref II). Thus, under conditions in which phosphotransfer in the Rcs system is strongly shifted towards phosphorylated RcsB, this system can quickly affect the synthesis of virulence factors at the post-transcriptional level by directly affecting the Rsm system. Interestingly, one of the RcsB binding sites in the *rsmB* regulatory region overlaps with the upstream activating sequence element known to be required for GacA(ExpA)-dependent expression of *rsmB* RNA structural homologues (Humair *et al.*, 2010). Whether phosphoactivated RcsB competes with the GacA response regulator for this site is currently under investigation.

1.2. The response regulator of the Rcs phosphorelay controls motility

In addition to the production of plant cell wall-degrading enzymes, motility has been shown to contribute to the virulence of pectobacteria. Because the Rcs phosphorelay has been shown to regulate the motility of many enterobacterial species, we studied the effect of the Rcs system on this trait in *P. wasabiae* (Clemmer & Rather, 2007; Krin *et al.*, 2010; Wang *et al.*, 2007). We found that the *P. wasabiae rcsB*-negative mutant is more motile in 0.3% agar than the wild type strain, whereas over-expression of *rcsB* strongly inhibits the swimming phenotype (Ref I).

Flagellum production in enteric bacteria is strictly dependent on the complex of FlhD and C proteins (McCarter, 2006). This complex acts as a positive regulator of flagellum synthesis by activating the transcription of a large set of genes coding for flagellar and chemotaxis proteins (Smith & Hoover, 2009). My results and those obtained in other enterobacteria show that RcsB directly represses the initiation of transcription from the *flhDC* promoter, and that is probably how it affects the motility of the bacterium (Francez-Charlot *et al.*, 2003, Ref II). The fact that wild type *P. wasabiae* is motile indicates that the repression of *flhDC* by RcsB is not completely suppressed under these conditions. However, activation of the phosphorelay may result in paralysis of the bacterium, as shown in *Salmonella enterica*, where a mutant with a constitutively active Rcs system is unable to swim or swarm (Wang *et al.*, 2007). Thus, one of the universal roles of Rcs signalling in enterobacteria is to optimize flagellum production in response to environmental conditions.

1.3. Conditions activating the Rcs system

Although the exact signal interacting with the RcsC input domain is not known, several conditions have been shown to activate the Rcs phosphorelay in enterobacteria (Clarke, 2010). In general, the conditions/compounds that regulate target gene expression in an Rcs-dependent manner can be taken together as modifiers of the cell envelope. Thereby it is generally accepted that Rcs system is activated in response to disturbances in the cell membrane or peptidoglycan layer. The sensing of cell envelope disturbance could certainly become important for bacteria in their natural habitats, where membrane integrity is of great importance. The mechanism by which the RcsC senses the membrane is not known. It is possible that the conformation of the sensor protein is directly affected by changes in the cytoplasmic membrane. The effect of a malformed cell envelope could also be indirect and mediated to RcsC by factors influenced by the stressors. For example, several signals are mediated to the sensor protein through outer membrane lipoprotein RcsF in *E. coli* (Callewaert *et al.*, 2009; Laubacher & Ades, 2008). In *P. wasabiae* we have shown that improper biosynthesis of the base of the flagellar structure, called the hook-basal-body (HBB), results in Rcs-dependent inhibition of extracellular protease production and necrosis-inducing protein synthesis (Laasik *et al.,* manuscript in preparation; data not shown). Similarly, studies conducted on *Salmonella enterica* demonstrate the relationship between Rcs system activation and incorrect assembly of the flagellar apparatus (Lin *et al.*, 2008). Thus it is tempting to speculate that as the Rcs phosphorelay is important for regulating flagellar motility, it somehow receives signals indicating improper assembly or absence of the flagellum and effects feedback control of flagellum synthesis.

According to my results the Rcs phosphorelay is constitutively active at a basal level under various conditions. This is corroborated by results showing that the Rcs mutants produce greater quantities of virulence factors on plates with both nutrient-rich medium and minimal medium supplemented with either glycerol or polygalacturonic acid (Ref I, Ref II). As in the regulation of motility, virulence factor synthesis is repressed at some level in various conditions. This accords with the fact that both these traits are regulated through synthesis of the FlhDC complex, the promoter of which can be bound by non-phosphorylated RcsB (Ref II). It remains to be discovered what conditions the bacterium meets in nature that lead to Rcs phosphorelay activation at a level causing the strongest decrease in flagellar and virulence gene expression. On the basis of current knowledge I hypothesise that the Rcs phosphorelay is important in surroundings that endanger the integrity of the pathogen's cell envelope. Under these conditions it may be important to remain sessile and use the available energy to protect the cell. Activation of the Rcs phosphorelay enables both; it represses flagellum production and activates synthesis of the protective capsule, later in *E. coli* and *Erwinia amylovora* (Gupte *et al.*, 1997; Wehland *et al.*, 1999). The repression of plant cell wall-degrading enzyme synthesis in *P. wasabiae* could be one way to conserve energy under hostile conditions.

2. The role of Rsm regulatory system in the *Pectobacterium wasabiae*

My results indicate that the Rcs phosphorelay is another signal transduction system that feeds into the Rsm regulatory system to control the production of virulence factors (Ref II). Because of the apparently central role of the Rsm system in regulating the virulence of *P. wasabiae*, our subsequent studies concentrated on investigating the role of RsmA.

RsmA homologues, constituting the CsrA protein family, are found in around 40% of the bacterial genomes sequenced to date (according to the Pfam database; Punta *et al.*, 2012). The majority of *rsmA* homologues are represented in the phylum *Proteobacteria* but examples can also be found in different phyla such as *Firmicutes*, *Spirochaetes,* and *Planctomycetes*. The number of *rsmA* homologues differs among individual bacterial genomes, reaching up to 10 copies in *Legionella drancourtii* and two species of *Planctomyces* (according to Pfam). However, with only a few exceptions, enterobacteria code for just one RsmA homologue. The *rsmA* gene has been shown to be essential in some enterobacterial species, whereas in *Escherichia coli* the importance of this gene depends on the conditions of bacterial growth (Altier *et al.*, 2000; Liaw *et al.*, 2003; Timmermans & Van Melderen, 2009). Nevertheless, *rsmA* knock-out strains have been successfully constructed in *P. c.* ssp. *carotovorum* and by us in *P. wasabiae* (Chatterjee *et al.*, 1995; Ref III).

The *rsmA*-negative strain in *P. wasabiae* is strongly disabled in proliferation, emphasising the importance of the RsmA protein in the functioning of the cell (Ref III). To study the genes regulated by RsmA in pectobacteria, we subjected the *rsmA*-negative and the wild type strains to gene expression profiling (Ref III). We found that 39% of the 4571 ORFs analysed were affected by the inactivation of *rsmA* (Fig. 4, Ref III). The differences in proliferation between the mutant and wild type could account for some of these changes. Two measures were taken to eliminate such genes from the analysis. First, samples from *rsmA*-negative cells were taken at different time-points. Secondly, our study relied on results confirmed indirectly by a previously-published microarray (Liu *et al.*, 2008, Ref III).

According to the microarray data and our subsequent studies, RsmA affects traits such as glycogen synthesis, transport of citrate, functioning of the TCA cycle, butanediol fermentation and many other cellular functions (Ref III). Details of the microarray results can be found from Ref III included in this thesis, but the following paragraphs discuss some of the functions of RsmA discovered in *P. wasabiae*.

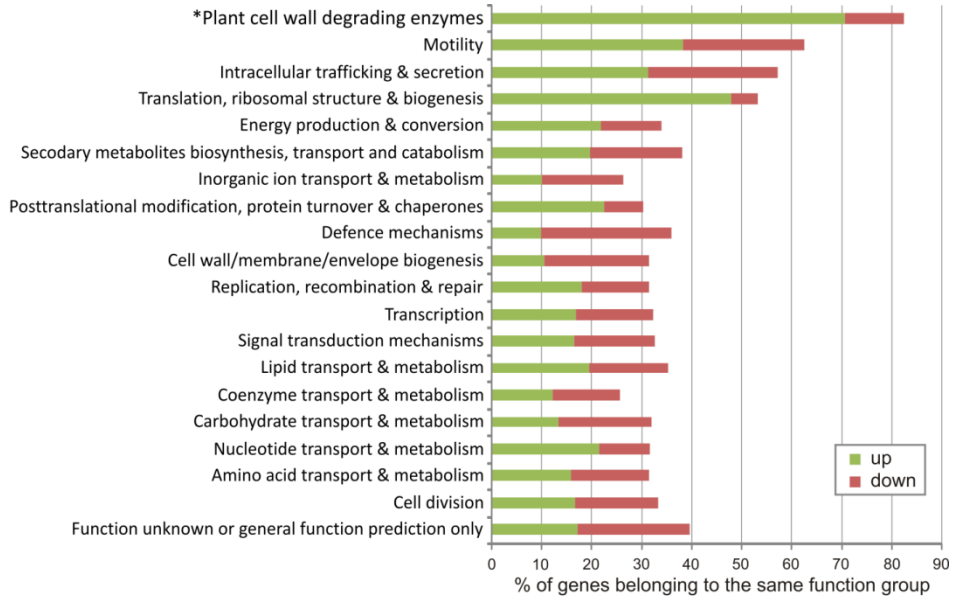

Figure 4. Genes affected by *rsmA* inactivation in *Pectobacterium wasabiae*. The mRNA profiles of the wild type and *rsmA*-negative strains were compared after growth of the respective strains on plates containing potato extract. The total RNA of the wild type strain was extracted after 24 hours of growth, and the RNA from the *rsmA*-defective strain was isolated 16 hours later because this strain proliferates more slowly. Bars indicate the percentages of genes belonging to different clusters of orthologous groups (COGs) according to the annotation supplied with the *P. wasabiae* genomic sequence, up- or down-regulated in the *rsmA*-negative strain according to the micro-array results (Koskinen *et al.,* in press; Ref III). The star indicates that the plant cell wall-degrading enzyme genes do not form a separate COG and the bar is constructed by subtraction of known or putative plant cell wall-degrading enzymes from the group comprising genes with unknown function or with general function prediction only.

2.1. Lack of RsmA results in constitutive virulence factor production

According to gene expression profiling, the plant cell wall-degrading enzyme genes are more highly expressed in the *rsmA*-defective strain than the parent strain of *P. wasabiae*. As the microarray results give information about the production of these enzymes on the mRNA level, we confirmed that the levels these virulence factors were elevated by measuring the enzyme activities in cell-free supernatants of the *rsmA*-defective and the wild type strains. The results showed that the *rsmA*-negative strain indeed produced significantly more pectinases and cellulase in the growth medium supernatant. As the plant cell wall-degrading enzymes are the main virulence factors in pectobacteria, their overproduction will lead to a more aggressive attack on the host plant. This was also true in the case of the *rsmA* mutant: it caused more severe disease

symptoms in potato tubers and had a higher infection rate in tobacco seedlings (Ref III). Although these results concerning *P. wasabiae* are novel, a hypervirulent phenotype underpinned by the massive production of extracellular enzymes in an *rsmA*-negative strain was demonstrated in the *Pectobacterium* genus in 1996 (Mukherjee *et al.*, 1996). Nevertheless, our microarray data together with studies of virulence factor production and tests for virulence contribute important new knowledge to this subject.

First, we demonstrate that the production of plant cell wall-degrading enzymes, which are known to be induced in response to oligogalacturonides, remained insensitive to these compounds in the *rsmA*-negative mutant. It is therefore possible that the effect of oligogalacturonides or even the host plant itself is strictly dependent on the liberation of RsmA repression. Alternatively, the induction could be masked by the endurance limit of pectinase production attained in the *rsmA*-negative strain.

Secondly, the production of plant cell wall-degrading enzymes was constitutively high in the absence of RsmA, whereas in the wild type strain the production of these enzymes was growth-phase-dependent. It is therefore likely that this repressor is necessary in the early stages of infection but its effect is less pronounced in later stages. This correlates well with the current model, where the extent of virulence factor production is controlled in response to bacterial population density (Kõiv & Mäe, 2001). It has been shown that *rsmA* expression is negatively controlled in response to the cell density signal, so it is likely to decrease as the population grows. Although *rsmA* expression has not been shown to decrease during infection of the plant, I hypothesise that it is one of the factors securing the accretion of plant cell wall-degrading enzymes in the course of disease development.

Thirdly, according to our microarray data, expression of not only the plant cell wall-degrading enzymes but also other genes, products of which are involved in virulence function, were up-regulated in the *rsmA* mutant of *P. wasabiae*. These include virulence protein Svx, necrosis-inducing protein Nip, and proteins related to the type VI secretion system (Corbett *et al.*, 2005; Mattinen *et al.*, 2004, 2007). What the corresponding genes have in common with the plant cell wall-degrading enzyme genes is that they are all produced in response to signals received from the host plant in *P. atrosepticum* (Mattinen *et al.*, 2007). This allows us to infer that the Rsm system acts as a common switch for controlling the expression of a set of host-induced genes, including the plant cell wall-degrading enzymes. Whether the signals inducing the expression of these genes are oligogalacturonides acting through the KdgR-Rsm pathway, or other compounds inducing different signalling systems feeding into the Rsm regulatory system, is yet to be discovered.

2.2. RsmA regulates motility genes

Another set of genes up-regulated in the *rsmA* mutant strain according to our microarray analysis are responsible for the biosynthesis and functioning of the flagellar apparatus (Ref III). The flagellum is an organelle required for bacterial locomotion. Depending on the physical properties of the medium, the bacterium uses different types of movement. To study the physiological effect of up-regulation of flagellar genes on motility, we tested the *rsmA* mutant for two types of flagellum-dependent motility: swimming and swarming.

Swimming motility, which is important for the bacterium in aqueous environments, is usually tested in low agar concentration media, where the pores are large enough to allow free movement of individual cells but form a network dense enough to increase the time required for the bacteria to spread away from the site of inoculation. This allows the swimming abilities of different bacterial strains to be compared visually. Wild type *P. wasabiae* is an excellent swimmer according to the motility test in the low agar concentration medium (0.25%), whereas the *rsmA*-negative strain could not move away from the inoculation site in this test (Ref III). The result was surprising for two reasons: first, our microarray data had shown higher expression of flagellar genes in the *rsmA*-negative strain; secondly, it has been shown previously in *P. c.* ssp. *carotovorum* that RsmA represses motility, so its removal should result in increased motility (Chatterjee *et al.*, 2010). We therefore decided to study the movements of individual cells under the light microscope. This showed that the *rsmA*-negative strain had no visible defect compared to the wild type in liquid media (Ref III). Additional motility tests indicated that the inability of the *rsmA* mutant to swim disappeared in certain media. Therefore the sessile phenotype recorded on the swimming plates was probably related to chemotaxis rather than the production of flagella.

Another type of bacterial motility requiring the synthesis of flagella is swarming. Unlike swimming, which occurs in liquids and is the movement of individual cells, swarming entails the formation of multicellular bundles that are capable of migrating over surfaces (Kearns, 2010). When we tested the swarming motilities of the *P. wasabiae* wild type and *rsmA*-negative strains we noted two things: firstly, *P. wasabiae* needs very specific conditions, including the presence of host-borne compounds, to initiate swarming; secondly, the *rsmA* mutant strain required significantly less time for the onset of swarming (Ref III), indicating that the RsmA protein represses this type of motility. The onset of swarming is known to depend on the length of the "swarm lag", a period of time during which the bacterium adjusts its cell physiology and morphology until it is suited for swarming. Swarmer cells differ in appearance from swimmers by becoming hyperflagellated and filamentous during the swarm lag (Kearns, 2010). We therefore suspected that as the *rsmA*-negative mutant starts to swarm significantly earlier than the parent strain, it does not need so much time to differentiate into swarmer cells. Maybe the lack of *rsmA* results in cells already equipped for swarming behaviour.

To study the morphology of the strains under study, we stained the cells together with flagella from different conditions and examined them under the light microscope. The *rsmA*-negative mutant cells were indeed elongated and had more flagella per cell under the conditions used for the microarray analysis (Ref III). This explains the higher mRNA expression levels of flagellar genes in our analysis. In contrast, *rsmA*-defective cells in liquid medium were rod shaped and flagellated like the wild type, so the mutant is not locked in the swarmer cell state and requires the presence of plant extract for swarmer cell differentiation. The wild type strain differentiated into swarmer cells under two conditions: on the plates used for swarming (0.4% agar plates containing potato extract) and in the potato tubers (Ref III). In animal pathogens the onset of the swarming phenotype is co-regulated with several virulence traits characteristic of the infectious form of the pathogen (Allison *et al.*, 1992; Givskov *et al.*, 1995; Macfarlane *et al.*, 2001; Senesi *et al.*, 2002; Walker *et al.*, 1999). Our findings demonstrate that this is also true for a plant pathogenic bacterium.

2.3. RsmA, translation and synthesis of ribosomes

The mechanism by which RsmA homologues regulate gene expression is known from previous studies. These regulators have a proven role in excluding a set of genes from translation; RsmA and its homologues physically block ribosome binding to the translation initiation sites to target mRNAs and accelerate their degradation (Romeo *et al.*, 2012). In this way, RsmA acts mainly as a negative regulator of translation initiation in bacteria[3]. The range of target genes of which the expression is directly affected by RsmA seems to be extremely wide. This is supported by the recent study by Edwards and coworkers, who have reportedly co-purified more than 700 different mRNAs using CsrA (an RsmA homologue) from *Escherichia coli* cells, and there are potentially more to catch as the mRNA pool they have sequenced was not saturated (2011). Although it needs to be verified whether the mRNAs of all the genes co-purified with CsrA really are direct targets for this repressor, the results clearly indicate that RsmA homologues are truly global regulators in the sense that they directly affect the expression of hundreds of genes in bacteria.

Supposing that RsmA also controls the translation of large set of genes in *P. wasabiae*, the removal of active RsmA from cells would result in the translation of genes otherwise repressed under these conditions. The occurrence of this phenomenon is indicated by the excessive production of dispensable proteins by the *rsmA* mutant strain; for instance, as noted earlier, higher levels of plant cell wall-degrading enzymes and flagellar components are synthesized in the *rsmA*-negative strain than the wild type strain (Ref III). At the same time, redundant protein synthesis has been demonstrated to inhibit bacterial growth (Kurland & Dong, 1996; Scott *et al.*, 2010). Uncontrollable translation of unnecessary genes

[3] With only one known exception, where it has been shown that an RsmA homologue enhances the translation of *flhD* mRNA in *E. coli* (Wei *et al.*, 2001).

could therefore be one reason for the poor growth of the *rsmA*-negative strain of *P. wasabiae*; the excessive protein synthesis consumes energy[4] and amino acids, and at the same time the translation of mRNAs excluded from translation in the wild type strain decreases the rate of translation of necessary genes. The latter might explain why the ribosomal protein genes are up-regulated in the *rsmA*-minus strain; I hypothesise that the mutant is trying to compensate for the shortage of active ribosomes (Fig. 4, Supplementary material in Ref III). Although we have not yet compared the numbers of rRNAs or ribosomes in wild type *P. wasabiae* and the *rsmA*-negative strain, I expect them to be higher in the mutant. In addition, we have seen that the *rsmA* minus strain is more resistant to the translation-inhibiting antibiotic streptomycin than the wild type strain (Fig. 5). Whether this effect is connected to the potentially higher ribosome number in the mutant is currently just an educated guess.

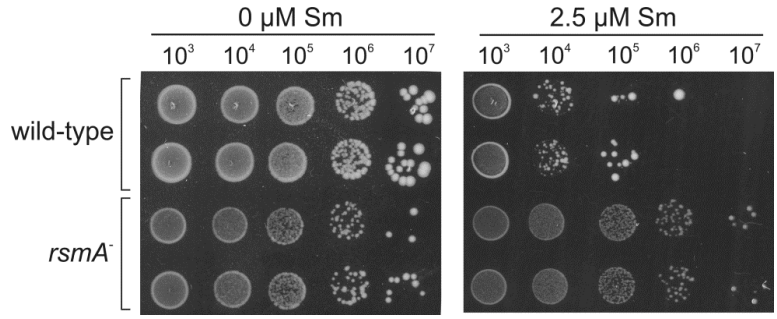

Figure 5. The RsmA-defective strain shows enhanced resistance to streptomycin. Dilution series of wild type and *rsmA* mutant stationary phase cultures were spotted on LB medium with (right) or without (left) the translation inhibiting antibiotic streptomycin and grown for 24 hours at 30°C. Numbers above the columns indicate the respective dilution factors.

Taken together, our microarray results suggest multiple roles for RsmA in the infection process and in the normal functioning of the cell. Thus, the Rsm system in pectobacteria not only has a role in the regulation of virulence factor synthesis but is also involved in the control of cellular physiology.

3. AepA in Pectobacterium

As the Rsm system is the centre of the regulatory network controlling virulence in pectobacteria, we were interested to identify the signals affecting the levels of Rsm system components. It was previously established that the quorum sensing signal as well as pectin degradation products enhance the production of

[4] Our microarray results indicate energy-deficiency in the *rsmA*-negative strain (Ref III).

virulence factors via the expression of *rsmA* and *rsmB*, respectively (Kõiv & Mäe, 2001; Liu *et al.*, 1999). However, the signal activating a phosphorelay that is seemingly very important in virulence regulation in pectobacteria, GacS/GacA (ExpS/ExpA), was not known. To study the signal that activates Gac-dependent transduction, we investigated the gene *aepA*, for two reasons: first, the virulence phenotype of the *aepA*-negative mutant in *P. c.* ssp. *carotovorum* is reportedly similar to the *gacA* (*expA*) mutant of our strain, since both produce very low amounts of virulence factors (Eriksson *et al.*, 1998; Liu *et al.*, 1993); secondly, the *aepA* product belongs to the family of amidohydrolases, which are known to catalyze a wide array of reactions involved in multiple cellular processes. Thus, we created a hypothesis according to which the product of *aepA* is involved in the synthesis of the signal molecule that interacts with the GacS (ExpS) input domain, and through the GacA (ExpA) response regulator activates the synthesis of *rsmB* RNA. To investigate the possible connection between *aepA* and the Gac system we constructed *aepA*-negative strains of *P. wasabiae* and *P. atrosepticum* (Ref IV). Surprisingly, these mutants produced exoenzymes at a similar level to the wild type strain, implying that *aepA* is not involved in the production of virulence factors in the strains tested. This controversy about the phenotypes of *aepA* mutants in different strains might be vacuous. The non-virulent *aepA* mutant of *P. c.* ssp. *carotovorum* was first isolated in 1991 by the Chatterjee group using transposon mutagenesis, according to its reduced ability to synthesise a set of plant cell wall-degrading enzymes (Murata *et al.*, 1991). However, in those days, the exact transposon insertion site had not been precisely identified. Seven years later, the same group demonstrated that approx. 200 nt upstream of *aepA* lies a gene for a regulatory *rsmB* RNA (Liu *et al.*, 1998). The phenotype of the *rsmB* mutant in *P. c.* ssp. *carotovorum* is the same as for the previously isolated *aepA* mutant, and *rsmB*[5] over-expression restored the production of plant cell wall-degrading enzymes in the reported *aepA* mutant (Murata *et al.*, 1994). Thus, while we could not investigate the *aepA* mutant created in *P. c.* ssp. *carotovorum* by Chatterjee's group, we think that it is actually an *rsmB*-negative strain and it is likely that AepA has no role in virulence regulation in *P. c.* ssp. *carotovorum* either.

The *aepA*-negative mutant of *P. wasabiae* and the *Pectobacterium* specific for potato, *P. atrosepticum,* showed abilities to macerate potato tubers similar to those of the corresponding parental strains (Ref. IV). Thus, *aepA* is not needed for full virulence of the pectobacteria, at least in potato tubers. We have studied the role of this amidohydrolase-family gene product in acquiring nitrogen from various sources, but with no success (Ref IV, our unpublished data). The role and the substrate for the *aepA* product in *Pectobacterium* species remain to be discovered, and because this family of proteins has a large spectrum of functions in living organisms, a different approach is required.

[5] At this time, the *rsmB* RNA coding region was assumed to code for a small protein named AepH.

4. Perspectives and future challenges

The results discussed in this thesis greatly enhance our knowledge of the regulation of gene expression in pectobacteria, but also bring new challenges. In this section I put our findings into a broader context and indicate some of the challenges for the future.

In the field of signal transduction we demonstrated the role of the Rcs phosphorelay in the regulation in *P. wasabiae* virulence (Ref I). In addition to *P. wasabiae*, this signalling system has previously been shown to regulate the virulent phenotypes of different animal and plant pathogens (Bouchart *et al.*, 2010; García-Calderón *et al.*, 2005; Hinchliffe *et al.*, 2008; Wang *et al.*, 2009). The diseases caused by these enterobacteria are very different, and so are the host organisms they infect, so the known virulence factors produced by these bacteria are extremely variable. This raises the question: is there a common mechanism used by the enterobacterial Rcs signal transduction systems to control virulence? My results indicate that the Rcs system in *P. wasabiae* controls virulence genes through indirectly modulating the activity of RsmA (Ref II). RsmA homologues have been found in all the enterobacterial genomes sequenced to date, so the Rcs phosphorelay could use the same system for virulence regulation in other species. This is further supported by the fact that in many members of the *Enterobacteriaceae*, RsmA or its homologue CsrA is involved in virulence regulation (Romeo *et al.*, 2012). Moreover, as found in the course of my work on *P. wasabiae*, exactly the same phenotypic traits, far more specific than the regulation of overall virulence, have frequently been shown to be similarly affected by the Rcs and Rsm system. These include swimming and swarming motility (Krin *et al.*, 2010; Liaw *et al.*, 2003; Morgenstein & Rather, 2012; Wei *et al.*, 2001), biofilm formation (Ferrières *et al.*, 2009; Wang *et al.*, 2005), glycogen biosynthesis (Baker *et al.*, 2002; Wang *et al.*, 2012) and butanediol fermentation (Ref III; our unpublished data). Therefore I hypothesise that the effect of the Rcs system through the Rsm system is conserved amongst enterobacteria. Future studies will clarify whether this is the case.

Another mechanism that seems to be conserved in enterobacteria is the direct regulation of flagellar gene expression by Rcs signalling; it has been shown in representatives of different enterobacterial clades, and now also in *Pectobacterium*, that RcsB directly represses the transcription of *flhDC*, the mRNA of which serves as a template to synthesise the master activator of flagellar genes (Francez-Charlot *et al.*, 2003; Wang *et al.*, 2007). As the activities of phosphotransfer system response regulators depend on their state of phosphorylation, they can be used to fine-tune target gene expression. Thereby, activation of the Rcs phosphorelay, which causes the activation of phosphate flow in the direction of RcsC-RcsD-RcsB and results in higher levels of phosphoactivated RcsB, causes a strong repression of *flhDC* transcription. Lower levels of *flhDC* mRNA in turn cause decreased production of flagellar proteins and that makes the bacterium sessile. In contrast, the down-regulation

of phosphate flow in this phosphorelay results in higher levels of *flhDC* mRNA, higher production of flagellar proteins and a motile phenotype. It has been demonstrated previously that the swarming phenotype requires a transient up-shift in *flhDC* expression. Thus, the Rcs phosphorelay may be one of the signalling systems that informs the bacterium whether the environment is suitable for swimming or swarming and regulates flagellum production accordingly. This is corroborated by the observation that Rcs signalling has a role in regulating swarming motility in *Proteus mirabilis* (Clemmer & Rather, 2007). As flagellar motility has been shown to contribute to the virulence phenotypes of several pathogenic bacteria, the regulation of flagellum production might be another function that unifies the Rcs system in different species and its role in virulence.

Knowing the environmental signal that activates the particular signalling system helps to elucidate the purpose for which the system has evolved, the question in which we are most interested in when studying signalling systems. Identification of the signal molecules that bind the input domains of the sensor proteins is currently the weakest area in studies concerning phosphotransfer-dependent signalling. One of the biggest problems in this area is the lack of a good method for observing the direction and/or activity of phosphotransfer within the signal transduction system under *in vivo* conditions. Therefore we cannot be sure if the effect we see in response to a signalling molecule is truly related to the activation of phosphate flow through the signalling system components. In the course of my studies, I could find no specific signal or stimulus that activated the Rcs phosphorelay, but I did identify an RcsB binding site that required phospho-activation of the RcsB to be bound (Ref II). This binding site can be used to construct a test system that would allow the level of phospho-RcsB in *P. wasabiae* to be monitored and thus aid in studying the conditions/signals inducing the phosphorylation of this regulator. Although such a test system would further studies of the Rcs phosphorelay, it will be of no use in studying other signalling systems. For that reason, a method that would allow us to observe phosphate flow within any system would be highly appreciated.

The fact that many signalling systems including the Rcs phosphorelay regulate the virulence of *P. wasabiae* through the Rsm system illuminates the importance the RsmA protein for virulence regulation in this genus of pathogens. Although the direct regulon of RsmA is yet to be identified in pectobacteria, microarray analysis shows that many cellular functions that are up-regulated in response to the host plant are also up-regulated in the absence of *rsmA*. I therefore hypothesise that the Rsm system acts as a switch to transmute between the pathogenic form of the bacterium and the bacterium with a non-infective lifestyle. According to our results these two life forms can be distinguished by their cell shape and functioning (Ref III). It would be interesting to ascertain what triggers the Rsm-dependent differentiation of *Pectobacterium* during the infection process. Is it a plant compound or is it synthesised by the pathogen itself? Is it a combination of different signals that give permission to attack?

The Rsm system is the strongest of the currently known effectors of the virulence phenotype of pectobacteria, so it could be the most suitable target for development of antimicrobial compounds to prevent *Pectobacterium* infection in fields and storages. One possible route that could be used to activate RsmA, thereby repressing virulence, is to induce phosphate flow through the Rcs phosphorelay. Thus it is important to identify the signal that activates Rcs signalling. When it is identified, the effect of this compound against the pectobacteria and other enterobacterial pathogens would be worth testing.

CONCLUSIONS

This study was launched to increase knowledge about virulence regulation in plant-pathogenic pectobacteria. We discovered that upon host contact, *Pectobacterium wasabiae* does not only start to produce virulence factors, but also changes its cell morphology and physiology to transmute into the infectious form. The synthesis of virulence factors is therefore strongly connected to changes in the physiology of the bacterium. According to our studies the post-transcriptional regulator RsmA is the main switch controlling the transformation of non-infective *P. wasabiae* cells into the highly virulent, hyperflagellated and elongated cells characterising the attacking form of the pathogen. This virulence switch is in turn controlled by different environmental signals, informing the bacterium about the surroundings; is the pathogen in the host plant? Is the bacterial population number high enough to start the attack? These questions are answered with the aid of various signal transduction systems that are able to transform information from the surroundings into a controlled cellular response. In this thesis I described a role for the signal transduction system named the Rcs phosphorelay in *P. wasabiae*. Although we do not yet know the signal controlling this system, we demonstrated that the Rcs phosphorelay regulates virulence through the Rsm system. In addition, the response regulator RcsB represses the expression of flagellar genes, thereby coordinating the production of virulence factors with the spreading of the pathogen. We hypothesised that the gene product of *aepA* is also involved in virulence regulation in *P. wasabiae*, but our findings showed that this is not the case in *P. wasabiae* or *P. atrosepticum,* and probably not in the other *Pectobacterium* species either.

Research on the regulation of virulence factors in pathogens, animal or plant, is not just important for improving our overall knowledge on the complexity of life. It also provides valuable information that could enable strategies for confronting these pathogens to be created. Our results on virulence regulation in *P. wasabiae* therefore have the potential to help in combating soft rot disease in the future.

I will still have to discard the soft-rotting apple when confronted with it, but hopefully in the future there will be fewer diseased fruits and vegetables; not just because they have treated with toxic pesticides, but because safer ways to avoid the disease have been developed.

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SUMMARY IN ESTONIAN

Virulentsusgeenide regulatsioon *Pectobacterium* perekonda kuuluvates taimekahjurites

Toidutaimede riknemine võib kodumajapidamises põhjustada kerget frustratsiooni, kuid kui see protsess leiab aset põldudel või hoidlates, on tulemuseks suur majanduslik kahju. Saagi hävimise üheks põhjuseks võivad olla bakterite tekitatud taimehaigused. Üheks levinud haiguseks toiduks kasutatavatel taimedel on bakteriaalne märgmädanik, mida tekitavad *Pectobacterium* perekonda kuuluvad kahjurid. Märgmädanikuks nimetatakse taimehaigust seetõttu, et *Pectobacterium* perekonna liikmed nakatavad taimede mahlasemaid osi, põhjustades vee väljavoolu taimekudedest. Haiguse arenedes muudetakse taimekude vesiseks massiks ning lõpuks taim sureb. Nimetatud bakteriperekond on inimestele oluline kahel põhjusel: esiteks, on need bakterid võimelised nakatama väga paljusid erinevaid toiduks kasutatavaid aedvilju; teiseks, võib *Pectobacterium* perekonda kuuluvaid baktereid leiduda kõikjal maailmas. Seega on nende bakterite tekitatud summaarne kahju väga suur ja just sel põhjusel on *Pectobacterium* perekonda kuuluvad bakterid mitmete teadusuuringute objektideks. Teadlaste eesmärgiks on leida viis, kuidas meie toitu märgmädanikku põhjustavate bakterite eest kaitsta. Tänaseks on jõutud nii kaugele, et on välja töötatud geneetiliselt modifitseeritud kartulitaimed, mis on *Pectobacterium* liikide suhtes resistentsed. Siiski pole peamiselt üldise avaliku vastuseisu tõttu geneetiliselt modifitseeritud organismidele selliste taimede kasvatamine toiduks Euroopas lubatud, ja kuna ükski tänapäeval toiduks kasvatatavatest kartulisortidest ei ole märgmädaniku suhtes immuunne, üritatakse haiguse tekkimisest hoiduda peamiselt kartulimugulate säilitustingimusi reguleerides.

Bakterid ei nakata taimi sihiga meid ärritada või meie liiki välja konkureerida. Taimepatogeenid vajavad taime selleks, et energiat saada ja paljuneda. *Pectobacterium* liigid kasutavad taimeraku polüsahhariidide, pektiini ja tselluloosi lagundamiseks rakust välja toodetavaid ensüüme. Bakterile on need ensüümid olulised süsinikuallikate hankimisel, meie aga nimetame neid virulentsusfaktoriteks, kuna need on peamised faktorid, mida patogeen haiguse tekitamiseks kasutab. Virulentsusfaktorite tootmine ei ole bakterile igasugustes tingimustes kasulik ja seetõttu on virulentsusgeenide ekspressioon täpselt reguleeritud sõltuvalt bakterit ümbritsevast keskkonnast. Käesoleva doktoritöö eesmärgiks oli uurida regulaatoreid, mis osalevad virulentsuse regulatsioonis taimepatogeenis *Pectobacterium wasabiae.* Töö kirjanduse osa käsitleb üldisemalt molekulaarseid mehhanisme, mida bakterid keskkonna tunnetamiseks kasutavad ning täpsemalt sensoreid ja regulaatoreid, mis on teadaolevalt haaratud virulentsusfaktorite sünteesi regulatsiooni *Pectobacterium* perekonna taimepatogeenidel. Töö tulemuste ja arutelu osas käsitlen 4 teadusartikli tulemusi, mille valmimisel osalesin. Kahe uurimustöö tulemusel selgus, et RcsC, D ja B valkudest koosnev signaalirada surub maha *Pectobacterium wasabiae* virulentsusfaktorite sünteesi mõjutades otse kahe tuntud regulaatorgeeni

ekspressiooni (publikatsioonid I ja II). Kolmandas tööle lisatud artiklis uurime lähemalt ühe tähtsama virulentsusregulaatorvalgu RsmA rolli selles patogeenis. Töö käigus selgus, et RsmA ei ole mitte ainult virulentsusfaktorite sünteesi regulaator, vaid osaleb ka mitmetes teistes protsessides. Näiteks mõjutab *rsmA* geeni puudumine bakteri tsentraalset metabolismi, kääritamisvõimet, varuainete kogumist, voogamist ning muutusi raku morfoloogias. Lisaks Rcs signaaliraja ja RsmA valgu rolli uurimisele püüdsime selgeks teha *aepA* geeniprodukti osalust virulentsusfaktorite sünteesil (IV artikkel). Nimelt oli varem näidatud, et *aepA* geeni inaktiveerimine muudab *Pectobacterium* perekonna bakteri nakatamisvõimetuks (Murata *jt.,* 1991). Meie tulemustest selgus, et *P. wasabiae* nagu ka ühe teise *Pectobacterium* perekonda kuuluva taimepatogeeni, *P. atrosepticum aepA*-negatiivne mutant nakatas kartulit sama hästi kui metsiktüvi. Meil on alust oletada, et 1991. aastal tehtud *aepA* mutant ei olnud korrektne ning seetõttu järeldame, et *aepA* ei osale *Pectobacterium* perekonna bakterite virulentsuse regulatsioonis. Mis roll tal bakterirakus on, jääb hetkel välja selgitamata.

Patogeensete bakterite virulentsuse regulatsiooni uurimine on oluline, sest kui teame, kuidas signaliseerimisregulatsiooni mehhanismid toimivad, annab see meile võimalusi patogeeni nakatamisvõime maha surumiseks. Kui me suudame seda efektiivselt teha, kasutamata inimesele ohtlike ühendeid, päästame suure osa oma toidust.

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PUBLICATIONS

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List of publications:

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Andresen, L., Sala, E., Kõiv, V. and Mäe, A. 2010. A role for the Rcs phosphorelay in regulating expression of plant cell wall degrading enzymes in *Pectobacterium carotovorum* subsp. *carotovorum*. *Microbiology*, 156, 1323–1334.

Andresen, L., Kõiv, V., Alamäe, T., Mäe, A. 2007. The Rcs phosphorelay modulates the expression of plant cell wall degrading enzymes and virulence in *Pectobacterium carotovorum* ssp. *carotovorum*. *FEMS Microbiol Lett*, 273, 229–238.

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