QUORUM SENSING IN THE PLANT PATHOGEN
ERWINIA CAROTOVORA SUBSP. CAROTOVORA

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Academic dissertation

To be presented for public criticism, with permission of the Faculty of Biosciences,
University of Helsinki, in auditorium 2 of the Viikki Infocentre, Viikinkaari 11, Helsinki, on
June 5th, 2009, at 12 o’clock.
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ISSN 1795-7079
ISBN 978-952-10-5513-3 (paperback)
ISBN 978-952-10-5514-0 (PDF, online)

Yliopistopaino  
Helsinki 2009
Dedicated to my father for everlasting encouragement.
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This thesis is based on the following publications which are referred to by their Roman numerals in the text.


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**ABBREVIATIONS**

aa  amino acid  
acyl-ACP  acylated acyl carrier protein  
AHSL  N-acylhomoserine lactone  
bp  base pair  
C  carbon  
Cel  cellulase  
CDS  coding sequence  
Eca  *Erwinia carotovora* subsp. *atroseptica* (*Pectobacterium atrosepticum*)  
Ecc  *Erwinia carotovora* subsp. *carotovora* (*Pectobacterium carotovorum*)  
Echr  *Erwinia chrysanthemi* (*Dickeya didantee*)  
GUS  β-glucuronidase  
HR  hypersensitive response  
HGT  horizontal gene transfer  
Hrp  hypersensitive reaction and pathogenicity  
Leu  leucine  
Met  methionine  
PCR  polymerase chain reaction  
PCWDE  plant cell wall-degrading enzymes  
Pel  pectate lyase  
Phe  phenylalanine  
Pme  pectin methylesterase  
Pnl  pectin lyase  
Prt  protease  
QS  quorum sensing  
ROS  reactive oxygen species  
RR  response regulator  
SAM  S-adenosyl-L-methionine  
Ser  serine  
SK  sensor kinase  
subsp.  subspecies  
T2SS  type II secretion system  
T3SS  type III secretion system  
TCS  two-component signal transduction system  
Thr  threonine
ABSTRACT

*Erwinia carotovora* subsp. *carotovora* (*Ecc*) is a Gram-negative enterobacterium that causes soft-rot in potato and other crops. The main virulence determinants, the extracellular plant cell wall -degrading enzymes (PCWDEs), lead to plant tissue maceration. In order to establish a successful infection the production of PCWDEs are controlled by a complex regulatory network, including both specific and global activators and repressors. One of the most important virulence regulation systems in *Ecc* is mediated by quorum sensing (QS), which is a population density -dependent cell-to-cell communication mechanism used by many Gram-negative bacteria. In these bacteria *N*-acylhomoserine lactones (AHSL), act as diffusible signaling molecules enabling communication between bacterial cells. The AHSLs are structurally diverse and differ in their acyl chain length. This gives the bacteria signaling specificity and enables the recognition and communication within its own species. In order to detect and respond to the AHSLs the bacteria use QS regulators, LuxR-type proteins.

The aim of this study was to get a deeper understanding of the *Ecc* QS system. In the first part of the study we showed that even different strains of *Ecc* use different dialects and of physiological concentrations, only the cognate AHSL with the “correct” acyl chain is recognized as a signal that can switch on virulence genes. The molecular basis of the substrate specificity of the AHSL synthase ExpI was investigated in order to recognize the acyl chain length specificity determinants of distinct AHSL synthases. Several critical residues that define the size of the substrate-binding pocket were identified. We demonstrated that in the ExpI<sub>SCC1</sub> mutations M127T and F69L are sufficient to change the *N*-3-oxohexanoyl-<i>L</i>-homoserine lactone producing ExpI<sub>SCC1</sub> to an *N*-3-oxooctanoyl-<i>L</i>-homoserine lactone (3-oxo-C8-HSL) producing enzyme. In the second study the means of sensing specificity and response to the AHSL signaling molecule were investigated. We demonstrated that the AHSL receptor ExpR1 of *Ecc* strain SCC3193 has strict specificity for the cognate AHSL 3-oxo-C8-HSL. In addition we identified a second AHSL receptor ExpR2 with a novel property to sense AHSLs with different acyl chain lengths. In the absence of AHSLs ExpR1 and ExpR2 were found to act synergistically to repress the virulence gene expression. This repression was shown to be released by addition of AHSLs and appears to be largely mediated by the global negative regulator RsmA.

In the third study random transposon mutagenesis was used to widen the knowledge of the *Ecc* QS regulon. Two new QS-controlled target genes, encoding a DNA-binding regulator Hor and a plant ferredoxin-like protein FerE, were identified. The QS control of the identified genes was executed by the QS regulators ExpR1 and ExpR2 and as expression of PCWDE genes mediated by the RsmA repressor. Hor was shown to contribute to bacterial virulence at least partly through its control of PCWDE production, while FerE was shown to contribute to oxidative stress tolerance and *in planta* fitness of the bacteria. In addition our results suggest that QS is central to the control of oxidative stress tolerance in *Ecc*.
In conclusion, these results indicate that *Ecc* strain SCC3193 is able to react and respond both to the cognate AHSL signal and the signals produced by other bacterial species, in order to control a wide variety of functions in the plant pathogen *Ecc*. 
A. INTRODUCTION

1. Plant pathogens

Plant diseases, insects and weeds account for an annual loss of 25-80% (40% for potato) in agricultural production worldwide (Oerke, 2006). In addition to fungi, which are the major causative agents of plant disease, prokaryotic and eukaryotic organisms such as bacteria, viruses, protozoa, nematodes and parasitic plants contribute substantially to agricultural losses. Infections caused by plant pathogenic bacteria account for approximately 14% of the lost crop globally (Agrios, 2005). Of the 1600 bacterial species known today, about one hundred, cause plant diseases with distinct symptoms such as leaf spots and blights, soft rots, wilts, and cankers. The most common bacterial plant pathogen genera include Agrobacterium (causing crown-gall disease), Clavibacter (potato ring rot, tomato wilt, fruit spot), Erwinia (blight, wilt and soft-rot), Pseudomonas (leaf spot, galls, wilt, blight and canker), Xanthomonas (leaf spot, cutting rot, canker and blight) and Streptomyces (potato scab and soil rot) (Agrios, 2005).

In order to cause plant infection bacteria need to enter the plant tissue. Most bacteria do this via stomates, wounds or by the help of feeding insects. After entering the plant the aggressiveness of the plant pathogen varies. Biotrophs multiply and can stay within the host tissue for a long time before killing it, while necrotrophs multiply fast in the host tissue and are capable of destroying it rapidly (Alfano and Collmer, 1996). Bacteria-plant interactions are highly coevolved and dynamic processes at molecular, cellular and colony-tissue level. Understanding the different strategies and mechanisms that bacteria use for plant infection will aid in the development of better means to defend plants from bacterial diseases (Alfano and Collmer, 1996).

2. The soft-rot pathogen Erwinia (Pectobacterium)

The bacterial disease soft-rot is caused by maceration of plant tissue by a massive amount of extracellular plant cell wall -degrading enzymes (PCWDEs) produced by the bacteria. This is symptomised by a water-soaked lesion that enlarges and develops to a slimy mass of disrupted cells and bacteria. One of the most common and destructive causatives of soft-rot are bacteria from the genus Erwinia, which has been renamed as Pectobacterium (Hauben et al. 1998). Throughout this thesis the former name Erwinia will be used. The genus Erwinia and the relatively closely related Pantoea includes both necrotrophic (including Erwinia carotovora subsp. carotovora, subsp. atroseptica, subsp. betavasculorum, subsp. odorifera, Erwinia chrysanthemi, Erwinia rhapontici, Erwinia cyripedii, Erwinia ananas and Erwinia carnegiena) and biotrophic bacteria (Erwinia amylovora and Pantoea stewartii) (Pérombelon and Kelman, 1980).
Different *Erwinia* species affect a wide variety of crops world-wide, from tropical to temperate regions. *Erwinia carotovora* subsp. *atroseptica* (*Eca*; recently renamed and reclassified as *Pectobacterium atrosepticum*; Gardan et al. 2003) has a narrow host range, restricted almost exclusively to potato in temperate regions, while *Erwinia carotovora* subsp. *carotovora* (*Ecc*; recently renamed and reclassified as *Pectobacterium carotovorum*; Gardan et al. 2003) has the broadest host range infecting a wide variety of crops in both subtropical and temperate regions (Pérombelon and Kelman, 1980; Toth et al. 2003). *Erwinia chrysanthemi* (*Echr*; recently renamed and reclassified as *Dickeya dadantii*; Samson et al. 2005) is most common in tropical and subtropical climates and has a wide host range including maize, pineapple, potato and African violet (*Saintpaulia ionantha*), which has been used extensively as a model host system.

Bacteria belonging to the genus *Erwinia* are Gram-negative, facultatively anaerobic, straight rods and motile with peritrichous flagella. The soft-rot *Erwinias* belong to the *Enterobacteriaceae* family and are thus related to human pathogens, such as *Escherichia coli* and *Salmonella enterica* serovar Typhimurium. Many of the methods developed for *E. coli* can therefore be utilized in *Erwinia* research (Barras et al. 1994). Comparison between the genomes of the plant pathogen *Eca* and enterobacterial human pathogens reveals that approximately three quarters of all CDSs are similar, while one quarter was either unique to *Eca* or similar to other plant pathogens (Toth et al. 2006). The unique part of the genome comprises the majority of the pathogenic determinants and it seems that many of these genes have been acquired by horizontal gene transfer (HGT) from non-enterobacterial plant associated bacteria. Acquisition of novel biological functions seems to be more influenced by physical rather than by phylogenetic proximity. The genomic data will aid in identifying what makes bacteria belonging to an *Enterobacteriaceae* family a plant or an animal pathogen and elucidate when these two classes have diverged evolutionary (Toth et al. 2006).

During its non-infective life-phase *Erwinias* can be found living as epiphytes and endophytes in plant and on their surfaces, as well as saprophytes in the soil and in groundwater. Little is known about the growth of *Erwinia* in these different environments. The recently obtained whole genome sequence of *Eca* suggests that *Erwinia* has the ability to respond to a wide range of nutrient sources and environmental cues, supporting the ability of *Erwinia* to exist in various habitats (Bell et al. 2004). Analysis of the *Eca* sequence data will shed more light on the less known non-pathogenic lifestyle and help to elucidate how *Erwinia* coordinates the onset of virulence processes after entering plants and how the transition from biotrophy to necrotrophy occurs. Latent infection of potato tubers is widespread and *Erwinia* cells can remain dormant for several months even as a large population. After entering a plant, *Erwinia* resides within the apoplastic region until environmental conditions, including free water, oxygen availability and temperature, become suitable for disease development (Toth et al. 2003). The main environmental factor triggering disease development is the presence of water, which leads to anaerobiosis and impairment of oxygen-dependent host resistance systems (Pérombelon, 2002). Disease tends to develop only when host resistance is impaired.
3. Virulence determinants of *Erwinia carotovora* subsp.

Successful infection by a plant pathogen requires coordinated production and secretion of various virulence factors. PCWDEs are considered the main virulence determinants of *Erwinia carotovora* subsp. In addition, several other factors and mechanisms such as motility, production of lipopolysaccharides, exopolysaccharides, harpins and other effector proteins, toxins, siderophores, Nep-I like proteins, factors protecting bacteria from oxidative stress and other antimicrobial defenses of the host contribute to the disease development (Barras *et al.* 1994; Toth *et al.* 2003; 2006). Recent analysis of the *Eca* genome revealed up to 393 coding sequences (CDSs) as putative pathogenicity genes with many novel factors in soft-rot *Erwinia* (Bell *et al.* 2004).

3.1. Plant cell wall-degrading enzymes

Soft-rot bacteria produce enzymes that degrade plant cell wall components in primary and secondary cell walls and middle lamella of plant cells. The degradation products are used as a source of energy and nutrients (Collmer and Keen, 1986; Barras *et al.* 1994). These enzymes are characteristically produced co-ordinately, rapidly and in large quantities, resulting in extensive tissue maceration and eventually death of the host plant. The PCWDEs include cellulases (Cel), proteases (Prt) and pectic enzymes (pectinases), such as pectate lyases (Pel), pectin methylesterases (Pme), pectin lyases (Pnl) and polygalacturonases (Peh) (Table 1).

Pectinases are the major enzymes involved in disease development by soft-rot *Erwinia*. Pectinases break down pectin in the plant cell wall and middle lamella into smaller subunits that can be transported into the bacterial cell and metabolized. *Erwinia* possesses several isoforms of Pels that are expressed by separate genes, possibly derived from successive rounds of gene duplication (Barras *et al.* 1987). The different isoforms of Pels cleave α-1,4-glycosidic linkages of pectin by β-elimination, either within the polysaccharide chain (endo-Pels) or from the ends (exo-Pels) (Collmer and Keen, 1986). Pathogenicity does not require activity of every type of Pel, indicating a wide substrate diversity and independent regulation. Pnls, known also as pectin methyltranseliminases, degrade both pectin and polygalacturonic acid (PGA), but only if PGA is methylesterified. Pnl activity is suggested to increase following exposure to plant-derived DNA-damaging agents (McEvoy *et al.* 1990). Unlike the other pectinases Pme is not a depolymerising enzyme, but instead removes methoxyl groups from pectin, producing polygalacturonic acid that can be broken down by other pectic enzymes such as Pehs. So far, only one gene encoding Pme has been characterized in *E. chrysanthemi*, though several *E. carotovora* strains have been shown to produce Pme activity.

Another class of PCWDEs are the Cels, which exhibit endoglucanase activity and break down cellulose in the primary and secondary cell walls of the host plant. In both *Ecc* and *Echr*, two different Cels that hydrolyze the β-1,4-glycosidic linkage of cellulose has been described. The Cels act in synergy with the other extracellular enzymes and are not essential for pathogenicity. However, inactivation of one of the Cels significantly reduced the
maceration capacity of potato tissue compared to wild-type *Ecc* (Walker et al. 1994). In addition, several proteases with a minor role in pathogenesis have been described. Their contribution to virulence is not yet fully understood, but could involve provision of amino acids for biosynthesis of bacterial proteins or degradation of host proteins associated with resistance (Toth et al. 2003). In *Ecc*, at least two, and in *Echr*, several proteases have been identified (Kyöstiö et al. 1991; Marits et al. 2002).

Table 1. The extracellular enzymes characterized in *E. carotovora* subsp. *carotovora.*

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Strain</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>Cellulases</td>
<td>CellA</td>
<td>LY34</td>
</tr>
<tr>
<td></td>
<td>CellB</td>
<td>LY34</td>
</tr>
<tr>
<td></td>
<td>CellS</td>
<td>SCC3193</td>
</tr>
<tr>
<td></td>
<td>CellV</td>
<td>SCC3193</td>
</tr>
<tr>
<td></td>
<td>PelB</td>
<td>SCRI193</td>
</tr>
<tr>
<td>Pectate lyases</td>
<td>PelA</td>
<td>EC</td>
</tr>
<tr>
<td></td>
<td>PelB</td>
<td>EC</td>
</tr>
<tr>
<td></td>
<td>PelI153</td>
<td>EC153</td>
</tr>
<tr>
<td></td>
<td>Pel-1</td>
<td>ECC71</td>
</tr>
<tr>
<td></td>
<td>Pel-3</td>
<td>ECC71</td>
</tr>
<tr>
<td></td>
<td>PelIII</td>
<td>ER</td>
</tr>
<tr>
<td></td>
<td>PelIII</td>
<td>ER</td>
</tr>
<tr>
<td></td>
<td>PelI</td>
<td>ER</td>
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<tr>
<td></td>
<td>PelX</td>
<td>ER</td>
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<tr>
<td></td>
<td>PelB</td>
<td>SCC3193</td>
</tr>
<tr>
<td></td>
<td>PelB</td>
<td>SCRI193</td>
</tr>
<tr>
<td></td>
<td>PelC</td>
<td>SCRI193</td>
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<tr>
<td>Pectin lyases</td>
<td>PnlA</td>
<td>ECC71</td>
</tr>
<tr>
<td></td>
<td>Pnl</td>
<td>ER</td>
</tr>
<tr>
<td>Polygalacturonases</td>
<td>Peh</td>
<td>EC</td>
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<tr>
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<td>ECC71</td>
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<td></td>
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<td>SCRI193</td>
</tr>
<tr>
<td>Proteases</td>
<td>PrtI</td>
<td>EC14</td>
</tr>
<tr>
<td></td>
<td>PrtW</td>
<td>SCC3193</td>
</tr>
</tbody>
</table>
3.2. Secretion systems

To accomplish a successful infection it is essential that the PCWDEs and other virulence determinants are secreted efficiently to the extracellular environment. The secreted proteins include both enzymes involved in attacking the plant tissue and those involved in more subtle interactions with the host plant, such as the effector proteins Svx (an AvrXca homolog of \textit{Xanthomonas campestris}) and Nep1-like proteins (a new family of well conserved proteins that induce an HR-like response) (Pemberton \textit{et al.} 2004; 2005; Mattinen \textit{et al.} 2004; Corbett \textit{et al.} 2005; Toth \textit{et al.} 2003; Toth and Birch, 2005). \textit{Ecc} possesses at least three secretion systems (type I-III) all of which function very differently and are conserved between different bacterial species. The type I secretion system is found to secrete metalloproteases in a single step process and seems to have a minor effect on virulence (Delepelaire \textit{et al.} 1991). The type II secretion system (T2SS) secretes pathogenicity determinants such as cellulases, pectinases, Svx and a Nep1-like-protein by a two-step mechanism. Proteins are exported to the periplasm by the Sec system followed by transport to the outside of the cell through a channel spanning the periplasmic compartment and the outer membrane, encoded by a 15 gene \textit{out} cluster (Johnson \textit{et al.} 2006). The importance of the T2SS in \textit{Ecc} is highlighted in a study where mutations affecting the T2SS resulted in an avirulent phenotype (Pirhonen \textit{et al.} 1991). The type III secretion system (T3SS) is described below.

Recently, based on genomic data the \textit{Eca} strain SCRI1043 was shown to contain at least three more independent secretion systems (type IV-VI) (Bell \textit{et al.} 2004). The type IV secretion system (T4SS) is used to translocate DNA and protein substrates, across the bacterial membranes to the target cell. The T4SS is used e.g. by the plant pathogen \textit{Agrobacterium tumefaciens} to deliver DNA into the host cell (Liu \textit{et al.} 2008). Proteins secreted by the type V secretion system are called autotransporters and are secreted by a two-step secretion process (Henderson \textit{et al.} 1998). The type VI secretion system is a newly described protein secretion system involved in virulence and conserved in several pathogens. In \textit{Eca} it was found to secrete potential effector proteins, such as Hcp and VgrG (Bingle \textit{et al.} 2008; Liu \textit{et al.} 2008).

3.2.1. The type III secretion system and effector proteins

The T3SS is present in many Gram-negative plant and animal pathogens. It consists of a long pilus/needle that is capable of penetrating the plant cell wall and translocates proteins into the host cell (Lahaye and Bonas, 2001). In plant pathogens, the system is used to secrete effector proteins that alter plant defense pathways by interacting with host cell components (Alfano and Collmer, 2004). Hypersensitive response (HR) is a defense-associated response in plants elicited by the effector proteins. In Gram-negative bacteria, the HR is elicited by products of the \textit{hrp} (hypersensitive response and pathogenicity) gene cluster that encodes the T3SS and the effector protein HrpN (harpin) that is translocated into the host plant by the T3SS (Alfano
and Collmer, 1996; Lahaye and Bonas, 2001). Genes encoding harpins have been reported from *E. amylovora* (Barny et al. 1990), *Eca* (Bell et al. 2004), *Ecc* (Mukherjee et al. 1997; Rantakari et al. 2001) and *Echr* (Bauer et al. 1995). The exact role of T3SS in *Ecc* is still unclear. *Ecc* does not normally elicit an HR in tobacco, but it can do so when HrpN is overproduced (Cui et al. 1996). The *hrp* gene cluster of *Ecc* was assigned a role in virulence when the *hrcC* gene encoding an outer membrane pore-forming protein was inactivated, delaying the bacterial growth during early stages of *Arabidopsis* infection (Rantakari et al. 2001). Nevertheless, a *hrpN* mutant retains wild-type maceration capacity in celery petioles (Mukherjee et al. 1997). Not all *Ecc* strains contain a *hrpN* gene. In a study, three out of eight *Ecc* strains tested contained the gene and the others lacked it, among them *Ecc* strain SCC3193 (Mattinen et al. 2004). In addition to the harpins, the effector protein DspA/E is translocated through the T3SS in plant pathogens. In *Eca* and *E. amylovora* the *dspA/E* operon is located adjacent to the *hrp* cluster (Holeva et al. 2004). Recently it was suggested that in *E. amylovora* HrpN plays a significant role in the translocation of DbsA/E into plant cells (Bocsanczy et al. 2008). DspA/E has been reported to manipulate the host defense by interacting with host proteins (Meng et al. 2006). It is widely conserved in phytopathogens and has been identified in *E. amylovora*, *Eca*, and *Echr* (DebRoy et al. 2004).

### 3.3. Other virulence determinants

#### 3.3.1. Motility

Motility is an important characteristic of plant pathogens in their natural habitat and many of them possess flagella. Motility appears to be necessary for *Erwinia* for successful invasion and infection of potato plants. Non-motile mutants of *Ecc* show reduced virulence, although they produce wild-type levels of PCWDEs (Pirhonen et al. 1991; Hossaina et al. 2005). Free water is essential for optimal disease development and may allow *Erwinia* cells to move more easily through the plant tissue (Mulholland et al. 1993). Motility may play an important role in contamination of tuber lenticels in wet soils (Pérombelon, 2002).

#### 3.3.2. Siderophores and tolerance to oxidative stress

Iron is an essential cofactor for many bacterial enzymes and is required for virulence in plant pathogens, but is often in short supply (Expert, 1999). Bacteria produce siderophores, which are chelating compounds that capture iron from the extracellular environment, as well as from host-secreted proteins. *Eca*, *Ecc* and *Echr* each have two siderophore systems (Bull et al. 1996; Franz et al. 1991; Ishimaru and Loper, 1992). Studies on siderophores have mainly focused on *Echr* (Expert, 1999). In *Erwinia*, siderophores are postulated to have a protective role, especially against toxic levels of iron that might lead to the generation of reactive
oxygen species (ROS) and oxidative stress (Expert, 1999). *Echr* mutants defective in siderophore-mediated iron transport remain localized, suggesting that siderophores also have a role in bacterial spreading throughout the plant (Enard et al. 1988).

One of the earliest pathogen induced defense responses is the oxidative burst, a rapid and transient production of large amounts of ROS at the site of attempted invasion (Doke, 1983; Wojtaszek, 1997). ROS promotes plant resistance to pathogens in several ways, it can be directly harmful to invading pathogens, but it also plays a central role in the development of host cell death during the HR reaction (Lamb and Dixon, 1997; Grant and Loake, 2000). Many virulence-associated genes protect bacterial cells from damage by ROS, including some that utilize iron (Nachin et al. 2001; Toth et al. 2003). Genes involved in oxidative stress tolerance, such as the *suf* operon, *sodA*, *msrA* and *ind* genes, have mostly been studied in *Echr* (Santos et al. 2001; Hassouni et al. 1999; Reverchon et al. 2002). In *Ecc*, a mutant defective in the general stress sigma factor RpoS was shown to be more sensitive to oxidative stress and exhibited reduced virulence *in planta* (Andersson et al. 1999a).

### 4. Virulence regulation in *Ecc*

Bacteria live in a constantly changing environment, where the conditions vary rapidly and unexpectedly. They have to respond to cues such as toxin and nutrient levels, acidity, temperature, oxygen and nitrogen availability, osmolarity, iron deprivation, various range of plant compounds, e.g. released by the action of the pathogen, DNA-damaging agents and the presence of other competing micro-organisms (Parkinson et al. 1995; Toth et al. 2003). Bacteria monitor their external milieu and adjust their gene expression pattern accordingly to these environmental factors. For successful plant infection bacteria have to circumvent or suppress the plant’s defense mechanisms and strictly control the production of pathogenicity factors. This in order to save metabolic energy and to hide from the plant until the bacterial population is large enough to overwhelm the plant defense. The strength of *Erwinia* is a co-ordinated production of large amounts of PCWDEs and other pathogenicity factors at critical stages of the infection process (Toth et al. 2003; Liu et al. 2008). This is a result of a sophisticated regulatory system, involving activators and repressors which regulate one specific target or act globally on multiple targets. Furthermore regulators also control expression of other regulators, forming a hierarchical and complex regulatory network.

### 4.1. Global activators and repressors

A key point in the *Ecc* virulence regulatory network is the *RsmA/B/C*-system (regulator of secondary metabolism; hereafter referred to as Rsm-system) (Figure 1). RsmA is an RNA-binding protein and acts as a repressor of a diverse set of pathogenicity-related functions such as production of extracellular enzymes and polysaccharides, flagellum formation and HrpN production. The *rsmA* gene is found in various strains of *Erwinia* (Cui et al. 1995; 1996;
Mukherjee et al. 1996). In *E. coli*, the RsmA homolog CsrA (carbon storage regulator) has been shown to bind the target gene mRNAs and thereby control their degradation (Liu and Romeo, 1997). Each of the known target gene transcripts contains multiple CsrA binding sites. Although considerable sequence variation exists among these RNA targets, GGA is the most highly conserved element (Dubey et al. 2005). A similar mechanism has been proposed for *Ecc*, where the transcript levels of *hslI* (gene encoding an acylhomoserine lactone synthase), *hrpN* and genes encoding PCWDEs were found to be higher in an *rsmA* mutant strain compared to wild-type *Ecc* (Chatterjee et al. 1995b; Cui et al. 1995; 1996).

RsmA is antagonized by interaction with the 5’ region of *rsmB* RNA, a regulatory non-coding RNA (formerly called *aepH*) (Murata et al. 1994; Liu et al. 1997; Liu et al. 1998). It is postulated that RsmA and *rsmB* act antagonistically to modulate the expression of genes that are expressed in a growth phase-dependent manner (Cui et al. 1999). The third component in this system is the RsmC protein (also known as HexY), a cytoplasmic protein that has been shown to increase *rsmA* expression and repress the transcription of *rsmB* (Cui et al. 1999). Therefore RsmC has a negative effect on PCWDE production (Cui et al. 1999). The RsmC protein has no known homologues among prokaryotes and was suggested to be unique to soft-rot *Erwinia* (Cui et al. 1999; Shih et al. 1999).

The Rsm-system is a target of many regulators and a central point in the regulatory network of *Ecc*. It is modulated by regulators such as KdgR, RpoS, ExpA and by the population density-dependent system called quorum sensing (QS) (Köiv et al. 2001; Chatterjee et al. 2002).

**Figure 1.** Schematic representation of the complex regulatory network involved in controlling PCWDE production in *E. carotovora* subsp. *carotovora*. Arrowheads indicate positive regulation and flattened ends denote negative regulation. Adapted and modified from Whitehead et al. 2002.
The global repressor KdgR is a DNA-binding protein and a member of the IclR family of transcriptional regulators (Thomson et al. 1999; Molina-Henares et al. 2006). KdgR from Echr has been characterized extensively (Hugouvieux-Cotte-Pattat et al. 1996). In Ecc, KdgR has been shown to negatively regulate the transcription of hrpN and genes encoding PCWDEs (Liu et al. 1999; Thomson et al. 1999; Hyytiäinen et al. 2001). The KdgR-mediated repression is relieved by the presence of plant cell wall degradation products such as 2-keto-3-deoxygluconate. KdgR has also been shown to modulate the expression of the Rsm-system. The expression of rsmA is under positive control of KdgR, while the expression of rsmB is under negative control (Liu et al. 1999; Hyytiäinen et al. 2001). A KdgR homolog, RexZ, has been identified in one Ecc strain. Although sharing substantial amino acid identity with KdgR, RexZ was found to be functionally different, acting as an activator of PCWDEs (Thomson et al. 1999).

Another modulator of the Rsm-system is the alternative sigma factor RpoS, a transcriptional regulator of a number of genes activated during the stationary phase and required for survival when nutrients are limited and during stress conditions (Venturi, 2003). In Ecc, RpoS acts as a negative regulator of extracellular enzyme production and hrpN expression. This has been shown to occur by RpoS positively regulating rsmA expression (Andersson et al. 1999a; Mukherjee et al. 1998). In addition RpoS acts as a positive regulator of oxidative stress tolerance in Ecc (Andersson et al. 1999a). Expression of RpoS is itself negatively regulated by ExpM, which is a response regulator identified in several Ecc species (Pirhonen et al. 1991; Andersson et al. 1999b; Mukherjee et al. 2000). The ExpM homologues of Salmonella (MviA) and E. coli (RssB/SprE) have been shown to regulate the stability of RpoS by acting as a recognition factor for the ClpXP protease during non-stress conditions (Andersson et al. 1999b; Hengge-Aronis, 2000). ExpM, on the other hand, is positively regulated by HexA, a DNA-binding LysR-type transcriptional regulator that negatively controls the production of PCWDEs through RsmA (Harris et al. 1998; Mukherjee et al. 2000). A HexA homologue PecT has been characterized in Echr (Hugouvieux-Cotte-Pattat et al. 1996). In some strains of Ecc HexA has also been shown to repress the production of the quorum sensing signal, N-acylhomoserine lactone, as well as the expression of hrpN and rsmB RNA (Harris et al. 1998; Mukherjee et al. 2000).

Also FlhDC, the master regulator of flagellar genes, was recently found to be a modulator of the Rsm-system (Cui et al. 2008). The flhDC operon, with strong homology to the E. coli flhDC, was shown to control extracellular enzyme production and hrpN gene expression in Ecc via GacA (ExpA) and HexA. In addition FlhDC was found to activate the expression of RsmC, though the extracellular enzyme production was not restored in the flhDC rsmC double mutant as could have been expected (Cui et al. 2008) (Figure 1).

Besides the RsmA dependent regulators of PCWDEs, Ecc also possess RsmA independent regulators, for example Hor (homology of rap), a DNA-binding protein belonging to the SlyA superfamily of regulatory proteins identified in several strains of Ecc. In Ecc, it is required for the full expression of extracellular enzyme genes and acts as an activator of the synthesis of the β-lactam antibiotic carbapenem (1-carbapen-2-em-3-
carboxylic acid) (Thomson et al. 1997; Holden et al. 1998). The production of carbapenem may protect the bacteria towards other microbes in a competitive environment. In *Serratia*, the Hor protein has also been shown to be regulated by QS (Fineran et al. 2005). Additional regulators affecting virulence in *Ecc* include the RdgA-RdgB system, activating Pnh in response to DNA damaging agents (McEvoy et al. 1992; Liu et al. 1997), CytR, a LacI-type regulator activating PehA and flagellum production (Matsumoto et al. 2003) and the activator AepA, a positive regulator of PCWDEs, which seems to be unique for *Erwinia* (Murata et al. 1991; Liu et al. 1993).

4.2. Two-component signal transduction systems

Bacteria use so called two-component signal transduction systems (TCSs) to respond to the environment and adapt their behaviour accordingly. A typical TCS includes a sensor kinase (SK) and a response regulator (RR). In a basic TCS, an external signal triggers the autophosphorylation of a SK, which subsequently phosphorylates the cognate RR. The phosphate group of SK is transferred to an aspartate residue on the RR, leading to DNA-binding of the RR and transcriptional activation of downstream genes (Parkinson, 1995). In addition to responding to a diverse set of environmental signals the TCSs can cross-regulate each other forming a network of functional interactions. The TCS have been most extensively studied in *E. coli*, which contains approximately 40 TCS (Oshima et al. 2002). The plant pathogen *Echr* is predicted to contain 30 genes that encode for different TCSs (Venkatesh et al. 2006). At least three TCSs, ExpS-ExpA, PehS-PehR and PmrB-PmrA, essential for virulence have been identified and described more extensively in *Ecc* (Eriksson et al. 1998; Frederick et al. 1997; Flego et al. 1997; 2000; Hyytiäinen et al. 2003).

The sensor kinase ExpS (GacS/RpfA) and the response regulator ExpA (GacA) form a TCS that is one of the key regulatory systems required for production of PCWDEs in *Ecc*. Consequently, an expA mutant is avirulent (Eriksson et al. 1998; Frederick et al. 1997). Homologous systems have been identified in a wide range of Gram-negative bacteria and studied mainly in *E. coli* (BarA-UvrY) and *Pseudomonas* sp. (GacS-GacA), where they control the synthesis of secondary metabolites, ecological fitness, stress tolerance and production of extracellular enzymes involved in pathogenicity in both plants and animals (Heeb and Haas, 2001). The input signal triggering the response is unknown, as are the ExpA target genes in *Ecc*. The ExpS-ExpA system is also a part of the Ecc regulatory network and has been shown to interact with the Rsm-system (Hyytiäinen et al. 2001; Cui et al. 2001). ExpA negatively regulates the expression of rsmA, but upregulates the expression of rsmB RNA (Liu et al. 1999; Hyytiäinen et al. 2001; Lapouge et al. 2008) (Figure 1).

The PehS-PehR TCS of *Ecc* responds to low levels of extracellular divalent cations such as calcium and magnesium and activates the production of one of the PCWDEs, the PehA during these conditions. On the contrary high levels (10 mM) of divalent cations repress the pehA expression (Saarilahti et al. 1992; Flego et al. 1997; 2000). In *Echr*, the PhoQ sensor kinase of the homologous PhoQ-PhoP TCS, was recently shown to involve
regulation of at least 40 genes encoding proteins involved in iron metabolism, membrane transport, stress responses, toxin synthesis and transcriptional regulators (Venkatesh et al. 2006). In Salmonella the TCS PhoQ-PhoP is considered the major virulence regulator with numerous target genes (García Véscovi et al. 1996; Groisman, 2001; Kato et al. 2008). Some of the PhoP-activated genes are also regulated by a PmrB-PmrA TCS. In Ecc, the components of this TCS are the SK PmrB and the RR PmrA. The PmrB-PmrA TCS responds to external changes in pH and to changes in iron and magnesium levels. The production of PCWDEs and the resistance to the antimicrobial peptide polymyxin B have been shown to be regulated by PmrB-PmrA (Hyytiäinen et al. 2003). A pmrB mutant showed highly increased resistance to polymyxin B and reduced expression of genes encoding for PCWDEs, while a pmrA mutant had wild-type resistance to polymyxin B and wild-type levels of PCWDEs. These differences in activity of PmrB and PmrA were suggested to be due to a negative autoregulatory mechanism exerted by PmrB (Hyytiäinen et al. 2003).

The TCSs PehS-PehR and PmrB-PmrA have been proposed to be involved in the following regulatory scenario: At the onset of infection the bacteria encounter an acidic plant apoplast with low concentrations of Mg$^{2+}$ and Ca$^{2+}$. This favours the PehR-controlled upregulation of PehA, which causes the initial damage to the plant tissue (Saarilahti et al. 1992; Flego et al. 1997). Coordinate activation of PehR-dependent low pH-inducible PmrA target genes are suggested to lead to modification of the cell surface properties, e.g. the lipopolysaccharides, and increased resistance to plant antimicrobial peptides. Subsequent plant tissue damage may lead to increased ion concentration in the apoplastic fluid, repressing the PehR-controlled genes and activating the PmrA target genes, including PCWDEs such as Cels, Pels and Prts (Hyytiäinen et al. 2003).

Following the multiplication of the bacterial population, one of the key virulence regulatory systems, QS, is activated. In a recent transcriptome study performed in Ecc, it was shown that several of the above mentioned regulators (RsmA, RsmC/HexY, KdgR, RexZ, Hor, RdgA, AepA, ExpA and PehR) are regulated by the QS system (Liu et al. 2008).
5. Cell-to-cell communication by quorum sensing

The QS system is a bacterial cell-to-cell communication system that uses an N-acylhomoserine lactone (AHSL) signaling molecule to co-ordinate group activities. An AHSL based QS system has been identified in more than 50 species of Gram-negative bacteria (Fuqua et al. 2002). However, *E. coli* and *Salmonella* do not possess AHSL synthases, but are still able to respond to the AHSL molecules with a LuxR-type protein called SdiA (Wang et al. 1991; Ahmer et al. 1998). *E. coli* and *Salmonella* possess another type of QS system that has been described in both Gram-negative and Gram-positive bacteria, which is based on an autoinducer-2 (AI-2) signaling molecule belonging to a family of furanones and synthesized by the LuxS protein (Vendeville et al. 2005). It has been suggested that AI-2 mediates universal and/or interspecies cell-to-cell communication. Additionally, Gram-positive bacteria have an own QS system based on oligopeptides. Here, the AHSL based QS system that is the best described and most common system in *Proteobacteria* is reviewed.

The QS phenomenon was originally discovered in the bioluminescent marine bacterium *Vibrio fischeri* in the early 1970s (Nealson et al. 1970; Eberhard et al. 1972). This bacterium colonizes the light organs of a variety of marine fishes and squids, where it occurs at a high population density (10^{10} cells/ml) and emits blue-green light. The emission of light was found to depend on an extracellular factor, an autoinducer, that regulates the synthesis of the enzyme luciferase (Eberhard et al. 1972). This autoinducer was later shown to be N-3-oxohexanoyl-L-homoserine lactone (3-oxo-C6-HSL) (Eberhard et al. 1981).

In a basic QS system the AHSLs are synthesized at a low basal level by an AHSL synthase, a LuxI-type protein first identified in *V. fischeri* (Figure 2) (Fuqua et al. 1994). These signaling molecules diffuse freely across the membrane out of the cell. As population density increases, the AHSL molecules accumulate and their concentration increase. When the concentration of AHSLs reaches a threshold level, a quorum, AHSL binds to a LuxR-type transcriptional regulator. Subsequently, this complex binds to a 20 bp DNA element of dyad symmetry, known as the *lux* box, modulating the target gene expression. In *V. fischeri*, target genes include the luminescence genes *luxICDABEG*, which are expressed at a higher rate in response to the LuxR-AHSL complex. The increase in the expression of *luxI* gene, encoding the AHSL synthase, establishes a positive feedback-loop of AHSL and luciferase synthesis, resulting in continuous emission of light (Figure 2).
Figure 2. The basic QS system demonstrated by the regulation of bioluminescence in *V. fischeri*. A. At low population density, expression of the genes for bioluminescence (*luxICDABEG*) is weak and insufficient for light emission due to low levels of AHSL. B. At high population density, a critical concentration of AHSL is reached. AHSL binds to LuxR and stimulates expression of the bioluminescence genes, leading to rapid amplification of the AHSL signal and light. Adapted and modified from Whitehead *et al*. 2001.

5.1. The AHSL signaling molecule and LuxI-type proteins

The AHSL signaling molecule (also called autoinducer-1, AI-1) is synthesized by the LuxI-type protein, the AHSL synthase. AHSL is composed of a fatty acyl chain ligated by an amide bond to a homoserine ring. The AHSLs are produced from the substrates S-adenosyl-L-methionine (SAM) and an acylated acyl carrier protein (acyl-ACP) with varying chain length. SAM binds to the active site of the AHSL synthase and the acyl group is then transferred from the appropriately charged acyl-ACP, forming an amide bond with the amino group of SAM. Subsequent lactonization of SAM results in formation of a homoserine ring and the final synthesis of AHSL (Figure 3) (Schaefer *et al*. 1996; Whitehead *et al*. 2001; Watson *et al*. 2002).
Figure 3. The schematic drawing illustrates the general features of the AHSL synthesis reaction. Two substrates, acyl-ACP and SAM, bind to the AHSL synthase. After the acylation and lactonization reactions, the product AHSL and holo-ACP and 5'-methylthioadenosine are released. Adapted from Watson et al. 2002.

There is considerable structural variety between AHSL signaling molecules from different bacteria and also between AHSLs synthesized by the same bacterium (Table 2). The acyl chain length can vary between four and eighteen carbons and the third carbon in the acyl chain may be a fully oxidized carbonyl, hydroxylated or be in the reduced state (Fuqua et al. 2002; Marketon et al. 2002). This variability is a result of the AHSL synthase’s specificity for the acyl chain substrate and may also be influenced by the available pool of acyl-ACPs (Fray et al. 1999; Watson et al. 2002). In P. aeruginosa, a series of fatty acid biosynthesis (fab) genes are responsible for the production of acyl-ACPs and were shown to be required for the synthesis of AHSLs (Hoang et al. 2002). The overall acyl chain length and chemical modification at the third carbon provide specificity to the distinct bacterial QS systems (Fuqua et al. 2001).

Different LuxI-type proteins have been identified from more than 50 Proteobacteria. They are 190-230 aa long and share 30-35% identity. Ten residues clustered in the N-terminal part are conserved within most LuxI-type proteins (Fuqua et al. 2002). The structure of two LuxI-type proteins, EsaI and LasI, from the plant pathogen P. stewartii and the opportunistic human pathogen P. aeruginosa, have been determined (Watson et al. 2002; Gould et al. 2004). The analysis show that the AHSL synthase share structural similarity with N-acetyltransferases and revealed, that an N-terminal electrostatic cluster of conserved charged residues is important for catalysis and integrity of the active site (Watson et al. 2002). Further the structural studies of EsaI and LasI, which generate 3-oxo-C6-HSL and 3-oxo-C12-HSL, respectively, showed that in LasI the acyl chain substrate bind to a V-shaped substrate binding cleft that leads to a tunnel binding the acyl chain substrate, without any restriction on length. The EsaI on the contrary possess a more closed hydrophobic pocket and therefore can only accommodate shorter acyl chain substrates (Watson et al. 2002; Gould et al. 2004).
In addition to the LuxI-type protein other AHSL-synthesizing proteins such as LuxM, AinS, VanM and HdtS have been identified in Vibrio sp. and P. fluorescens. These proteins however do not share significant sequence homology with the LuxI-type proteins (Bassler et al. 1994; Gilson et al. 1995; Laue et al. 2000; Milton et al. 2001; Watson et al. 2002).

Table 2. Examples of LuxI-LuxR-type QS systems.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Regulators</th>
<th>Chain length (R2)</th>
<th>β R-group (R1)</th>
<th>Target function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agrobacterium tumefaciens</td>
<td>TraI-TraR</td>
<td>C8 (4)</td>
<td>=O</td>
<td>Conjugal transfer</td>
<td>Zhang et al. 1993</td>
</tr>
<tr>
<td>Erwinia carotovora subsp. carotovora</td>
<td>ExpI-ExpR</td>
<td>C6 (2)</td>
<td>=O</td>
<td>Extracellular enzymes and antibiotics</td>
<td>Pirhonen et al. 1993; Jones et al. 1993; McGowan et al. 1995</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>LasI-LasR</td>
<td>C12 (8)</td>
<td>=O</td>
<td>Virulence and biofilm development</td>
<td>Pearson et al. 1997</td>
</tr>
<tr>
<td>RhlI-RhlR</td>
<td></td>
<td>C4 (0)</td>
<td>−H</td>
<td>Virulence and rhamnolipids</td>
<td>Pearson et al. 1997</td>
</tr>
<tr>
<td>Rhizobium leguminosarum</td>
<td>CinI-CinR</td>
<td>C14:1 (10)</td>
<td>−OH</td>
<td>Rhizome interactions, root nodulation</td>
<td>Lithgow et al. 2000</td>
</tr>
<tr>
<td>Sinorhizobium meliloti</td>
<td>SinI-SinR</td>
<td>C16:1 (12)</td>
<td>−H</td>
<td>Exopolysaccharide</td>
<td>Marketon et al. 2002</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C18:1 (14)</td>
<td>−H</td>
<td>Exopolysaccharide</td>
<td>Marketon et al. 2002</td>
</tr>
<tr>
<td>Vibrio fischeri</td>
<td>LuxI-LuxR</td>
<td>C6 (2)</td>
<td>=O</td>
<td>Bioluminescence</td>
<td>Eberhard et al. 1981</td>
</tr>
</tbody>
</table>

5.2. LuxR-type proteins

In order to detect and respond to the AHSL signaling molecule the bacteria use QS regulators, LuxR-type proteins. Members of the LuxR-type protein family are AHSL-responsive transcriptional activators or repressors. The LuxR-type proteins are approximately 250 aa in length and share a similar structure composed of two functional domains: an N-terminal domain involved in binding AHSL and a conserved C-terminal domain containing a helix-turn-helix motif involved in DNA-binding and subsequent transcriptional regulation (Nasser and Reverchon, 2007).

The LuxR homologs TraR and SdiA of A. tumefaciens and E. coli, respectively, are the only LuxR-type proteins that have been crystallized (Qin et al. 2000; Vannini et al. 2002; Zhang et al. 2002; Wu et al. 2008). The AHSL-binding cavity of TraR consists of a five-stranded antiparallel β-sheet with three α-helices on each side (Vannini et al. 2002). The
AHSL molecule was shown to be fully embedded within the protein, in a narrow cavity formed mostly by hydrophobic and aromatic residues (Vannini et al. 2002; Zhang et al. 2002). The amino acid composition in the AHSL binding cavity and the acyl side chain of the signaling molecule play an important role in the regulator’s affinity and specificity for its ligand. This specificity is essential for bacteria to distinguish between AHSLs synthesized by their own species and those synthesized by other species (Nasser and Reverchon, 2007).

5.2.1. Mechanism of action

The LuxI/R system of V. fischeri, the TraI/R system of A. tumefaciens, the LasI/R and RhlI/R systems of P. aeruginosa and the EsaI/R system of P. stewartii are the best characterized QS systems. Although LuxR-type proteins have similar structures, their functional mechanisms differ. Binding of AHSL to the QS regulator results in large conformational changes that lead to modification of the regulators transcriptional activity, acting either as activator or repressor (Figure 4) (Welch et al. 2000; Zhu et al. 2001).

In most of the LuxR-type activators, AHSL binding induces dimerization or multimerization of the protein, which leads to exposure of the C-terminal DNA binding site and subsequent binding to the target DNA. In the absence of AHSL, the N-terminal domain masks the C-terminal DNA-binding domain, preventing interaction with target genes (Nasser and Reverchon, 2007). TraR, LuxR, RhlR and LasR bind to their target DNA only in the presence of their cognate AHSL in dimerized form (Zhu et al. 2001; Lamb et al. 2003; Schuster et al. 2004a; Urbanowski et al. 2004). Biochemical analysis has revealed that all investigated LuxR-type proteins bind to their cognate AHSL with a stoichiometric ratio of one molecule of protein to one molecule of AHSL (Welch et al. 2000; Zhu et al. 2001; Schuster et al. 2004a; Castang et al. 2006). Qualitative differences have been reported concerning the regulator’s affinity for its ligand, as well as the stability of the ligand-regulator complex. The interaction of TraR with its AHSL ligand is strong and irreversible, while LuxR binds its ligand less tightly and in a reversible manner (Urbanowski et al. 2004).

Contrary to the LuxR-type activators, the LuxR-type repressors form active dimers in the C-terminus that bind to the target DNA in the absence of bound AHSL (Whitehead et al. 2001; Lazdunski et al. 2004). The presence of AHSL results in dimerization of the N-terminal domain, inducing a conformational change in the C-terminal region, which leads to dissociation of the repressor-DNA complex (Frezza et al. 2006). Examples of LuxR-type repressors are EsaR from P. stewartii (von Bodman et al. 1998; Minogue et al. 2002), ExpR from Echr (Nasser et al. 1998) and VirR/ExpR from E. carotovora subsp. (Andersson et al. 2000; Burr et al. 2005; Chatterjee et al. 2005; Cui et al. 2006). In the absence of bound AHSL, EsaR forms a functional DNA-binding homodimer, which represses the transcription of its target genes esoR and rscA, encoding an essential virulence regulator (Minogue et al. 2005). The interaction of EsaR with AHSL abrogates repression and allows the transcription of target genes. Consequently, an esoR mutant produces higher levels of the virulence factor exopolysaccharide compared to the wild-type (von Bodman et al. 1998).
**Figure 4.** Schematic presentation of the structural modifications induced by AHSL binding on the activators and repressors of the LuxR-type family. **A. Activator:** In the absence of AHSL, the N-terminal domain masks the C-terminal DNA-binding domain and thus interferes with the DNA binding. Binding of the appropriate ligand to the activator induce conformational changes that lead to exposure of the DNA-binding domain and subsequent DNA-binding. **B. Repressor:** In the absence of AHSL, the C-terminal domain is in a dimeric form, resulting in a repressor–DNA complex; the binding of the ligand to the N-terminal domain results in its dimerization, which interferes with the appropriate folding of the C-terminal region. This releases the repressor-DNA complex. Adapted and modified from Nasser and Reverchon, 2007.

The *lux* box DNA sequence consists of a 20 bp inverted repeat (Egland and Greenberg, 1999). Similar *lux* boxes have been identified in the DNA targets of many LuxR-type proteins. Different *lux* boxes contain subtle differences in their sequences, which determine the specificity between the regulator and the target gene. This is supported by studies performed on *P. aeruginosa* LuxR-type proteins LasR and QscR. The 20 bp *lux* boxes of LasR and QscR share 15 bp identities, however the binding of LasR and QscR to their target gene *lux* boxes are not interchangeable (Lee *et al*. 2006). In another study it was shown that EsaR of *P. stewartii* could bind both to its cognate *esa* box and to a non-cognate *lux* box, however the binding to the non-cognate *lux* box was fivefold weaker (von Bodman *et al*. 2003). The function of a regulator is also determined by the position of the binding site relative to the transcription initiation site of the target genes, because it influences the potential interaction of the regulatory protein with RNA polymerase (Egland and Greenberg, 1999; White *et al*. 2005; Nasser and Reverchon, 2007). The activators LuxR and TraR participate in recruiting RNA polymerase and interact with it, while there is no evidence for
interaction between a LuxR-type repressor and RNA polymerase. ExpR in Echr prevents access of RNA polymerase to the target gene, thereby preventing initiation of transcription (Castang et al. 2006). EsaR has also been shown to sterically block transcription initiation by RNA polymerase (Minogue et al. 2005).

5.3. Quorum sensing in the plant pathogen Erwinia carotovora subsp.

The QS system is widely used in plant-associated bacteria as a regulator of virulence. In a study where 106 isolated bacterial strains, representing seven bacterial genera associated with plants, were screened showed that most of the Agrobacterium, Rhizobium, and Pantoce isolates and about half of the Erwinia and Pseudomonas isolates were found to elicit positive QS reactions (Cha et al. 1998). In Erwinia carotovora subsp. QS is one of the key regulatory mechanisms of virulence. Disruption of the AHSL synthase gene in Ecc abolishes the production of PCWDEs, resulting in an avirulent phenotype of Ecc (Jones et al. 1993; Pirhonen et al. 1993).

The Ecc and Eca strains mainly synthesize two different types of AHSLs and have been divided into two groups based on these (Chatterjee et al. 2005). Class I, including Ecc strains SCC3193 and EC153, synthesize mainly N-3-oxooctanoyl-L-homoserine lactone (3-oxo-C8-HSL), while class II, including Ecc strains ATCC39048, GS101, AttHn10 and MS1, Ecc71 and Eca strain SCR1043, synthesize predominantly N-3-oxohexanoyl-L-homoserine lactone (3-oxo-C6-HSL) (Table 2) (Chatterjee et al. 2005; Barnard and Salmond, 2007). A single AHSL synthase is present in each of the different Ecc and Eca strains and are highly related. In ATCC39048 the synthase is called CarI (Swift et al. 1993), in Ecc71 AhII (Cui et al. 2005) or HsII (Cui et al. 1995), in SCC3193 ExpI (Pirhonen et al. 1993) and in the Eca strain SCR1043 it is ExpI (Bell et al. 2004). In addition to the AHSL molecules, Erwinia also produces AI-2, which requires the LuxS-protein for synthesis (Coulthurst et al. 2006).

In order to respond to the AHSL signaling molecule the Ecc and Eca strains possess one, two or three different LuxR-type QS regulators. In Ecc strain ATCC39048, the QS regulators are called CarR, EccR/ExpR and VirR (Swift et al. 1993; Bell et al. 2004), in Ecc71 and SCC3193 they are called ExpRs (Pirhonen et al. 1993; Chatterjee et al. 1995b; Andersson et al. 2000; Cui et al. 2006) and in the Eca strain SCR1043, the QS regulators are called ExpR and VirR (Bell et al. 2004; Burr et al. 2005). This variety in names reflects well the active research executed within the area of Erwinia QS.

5.3.1. Quorum sensing regulators of Ecc

All Erwinia carotovora subsp. strains that have been investigated possess at least one QS regulator encoding gene, expR1/eccR, located adjacent to the expI gene (Andersson et al. 2000; Barnard and Salmond, 2007). Most strains of E. carotovora subsp. also contain another QS regulator-encoding gene, expR2/virR, positioned elsewhere in the genome (Bell et al.
2004; Cui et al. 2006; Burr et al. 2005). These two QS regulators are closely related and have been shown to act as negative regulators of virulence by an indirect mechanism mediated via the global negative regulator RsmA (Andersson et al. 2000; Cui et al. 2005; 2006; Burr et al. 2005). This topic is part of the thesis and is described in detail in the Results and Discussion-section.

Another kind of mechanism is represented by the QS regulator CarR of Ecc strain ATCC39048, an activator that positively regulates the production of the β-lactam antibiotic carabapem. The carR gene is located immediately upstream of the carA-H biosynthetic operon, which encodes proteins required for carabapem biosynthesis and for the carabapem intrinsic resistance function (McGowan et al. 1995; Holden et al. 1998). Binding of 3-oxo-C6-HSL to CarR induces multimerization of the protein and subsequent binding of the complex to the carA promoter and activation of transcription of the carA-H operon (Welch et al. 2000). In addition the carR expression is tightly autoregulated, as generation of CarR is dependent on a CarR/3-oxo-C6-HSL complex (McGowan et al. 2005). Disruption of carI and/or carR represses transcription from the QS-dependent carA promoter and abolishes carabapem production (McGowan et al. 2005). Environmental factors such as temperature, pH and carbon source also affect synthesis of carabapem. The environmental influence on carabapem synthesis is at least to some extent mediated by QS, either by affecting carI transcription or the stability of the AHSL molecule (McGowan et al. 2005). The carA-H biosynthetic operon and the carR gene are only present in a few strains of Erwinia carotovora subsp. (Holden et al. 1998), while the AHSL signaling molecule is distributed widely as a major virulence regulator of Erwinia carotovora subsp. Inactivation of the two other LuxR-type QS regulators VirR and ExpR in Ecc strain ATCC39048 has no obvious influence on carabapem production (Barnard et al. 2007).

5.3.2. Quorum sensing controls a wide variety of functions

For long the Ecc QS system was primarily known as the central regulator of the production of the main virulence factors PCWDEs and the antibiotic carabapem (Jones et al. 1993; Pirhonen et al. 1993). Recent screening studies have revealed new targets of the QS system. In these studies the necrosis inducing protein, Nip (Mattinen et al. 2004; Pemberton et al. 2005), and the putative effector protein, Svx (Corbett et al. 2005), were shown to be part of the QS regulon. The QS regulon of Eca was further broadened in a transcriptome analysis performed in vivo in potato tubers (Liu et al. 2008). This analysis revealed that in the Eca strain SCRI1043 26% of the genome was differentially expressed in an expI mutant compared to wild-type. This newly identified QS regulon includes type I, type II, type III secretion systems and a novel type VI secretion system along with several secretion system substrates and more than 70 regulators, of which many are known regulators of pathogenesis (HexA, KdgR, PehR, RdgA, RsmA, AepA, ExpA, ExpR, HexY, Hor, RexZ, VirR). In addition novel virulence determinants, such as the proteins CFA2 and CFA8, involved in the synthesis of coronafacic acid, a putative suppressor of plant defense, were also found to be part of the QS
regulon (Liu et al. 2008). Similar results were obtained in a transcriptome analysis performed in *P. aeruginosa*, which showed that QS regulates at least 300 genes directly or indirectly (Schuster et al. 2003; Wagner et al. 2003). It is still not clear whether the newly identified QS target genes in *Eca* are regulated directly or indirectly by the QS regulators. Only the carbapenem biosynthesis cluster and the *rsmA* have been shown to be direct targets of the QS regulators in *Ecc* (Welch et al. 2000; Chatterjee et al. 2005).

### 5.3.3. Cross-talk between quorum sensing and the regulatory network

QS is a central regulator of virulence and essential for successful plant infection. However, *Erwinia carotovora* subsp. also possesses many other essential regulators, which together with QS form a complex and hierarchical regulatory network. The regulators are connected to QS either directly or indirectly. It has been suggested that the Rsm-system acts as a central input point for the regulators (Figure 1) (Barnard and Salmond, 2007). As QS also acts via RsmA, other regulators such as ExpA, RpoS and KdgR, acting at this key point also have implications on the QS regulon (Barnard and Salmond, 2007). Recently it was shown that the expression of KdgR is also regulated by QS (Liu et al. 2008). Similarly to QS, KdgR plays an important role in the timing and fine tuning of virulence regulation, as KdgR represses the production of PCWDEs until plant cell wall degradation products are available. Cross-talk between QS and other regulators has also been observed in e.g. *P. aeruginosa*, where both RpoS and the GacA/GacS TCS (homologous to ExpA/ExpS) were found to modulate the expression of numerous QS controlled genes, either directly or indirectly via control of the LuxR-type regulators LasR and RhlR (Chancey et al. 1999; Pessi et al. 2001; Heurlier et al. 2004; Schuster et al. 2004b).

Recent findings suggest that QS is essential along the whole infection process, starting at the onset of plant infection when the initial, small *Erwinia* population becomes subject to the host’s defense system. It has been proposed that the QS controlled T3SS may hinder or delay the plant defense response, giving the bacteria time to multiply before starting the production of PCWDEs (Rantakari et al. 2001; Toth et al. 2005; Liu et al. 2008). As PCWDEs themselves are potent activators of plant defense responses through release of plant cell wall fragments, they need to be produced in large amounts to overcome the plant’s defense mechanisms, which in turn require a high bacterial population density (Palva et al. 1993; Salmond et al. 1995; Måe et al. 2001; Barnard and Salmond, 2007). The QS system has been suggested as a master regulator of phytopathogenesis and is placed high in the regulatory hierarchy (Liu et al. 2008).
5.4. Quorum quenching

*Erwinia* mutants with defective QS have highly reduced virulence, confirming the phytopathological significance of the phenomenon. Therefore, it is suggested that strategies that impede the QS system are useful for therapeutic and prophylactic applications (Whitehead *et al.* 2002). Multiple potential targets for such interventions have been suggested.

Generation of a transgenic host plant that expresses AHSL lactonase or AHSL acylase that can degrade the AHSL signaling molecule could be used for reducing AHSL levels. Such enzymes have been identified in a range of bacterial species, and transgenic potato and tobacco plants that express AiiA, an AHSL lactonase from *Bacillus cereus*, have been generated. These transgenic plants reduced AHSL signaling activity and were highly resistant to infection by *Ecc* (Dong *et al.* 2000; 2001; Zhang, 2003). In addition, a study where AHSLs were produced in transgenic plants, expressing a LuxI-type protein, resulted in enhanced resistance to infection by *Ecc* (Mäe *et al.* 2001). This was suggested to be due to premature induction of PCWDE production at low *Ecc* population density, resulting in a successful plant host defense response (Palva *et al.* 1993; Mäe *et al.* 2001; Whitehead *et al.* 2001). However, in another study contrary results were observed, showing that transgenic tobacco plants producing AHSL molecules lead to increased disease development (Toth *et al.* 2004).

Another approach involves the use of AHSL mimics, such as halogenated furanones. The furanones are structurally similar to the AHSLs and are suggested to bind to LuxR-type proteins, thereby preventing the binding of AHSLs by competitive inhibition and initiating turnover of the LuxR-type protein (Manefield *et al.* 1999; 2002). Compounds similar to the halogenated furanones that were originally isolated from the red algae *Delisea pulchra* interfere with QS of many micro-organisms (Givskov *et al.* 1996). Molecules that mimic the QS signal have also been identified from pea, rice, tomato, soybean and *Medicago truncatula* (Teplitski *et al.* 2000). In the legume *M. truncatula* 150 proteins are controlled in response to the AHSLs produced by *Sinorhizobium meliloti* and *P. aeruginosa*. The results indicated that eukaryotes have an extensive range of functional responses to AHSLs that may play important roles in the beneficial or pathogenic outcomes of bacteria-host interactions (Mathesius *et al.* 2003). Further AHSLs have been shown to induce defence responses in tomato and were suggested to function as microbe-associated molecular patterns (MAMPs) (Boller and Felix, 2009). *Serratia liquefaciens* and *P. putida* that colonize tomato roots and produce AHSLs in the rhizosphere were shown to increase systemic resistance of tomato plants against the fungal pathogen *Alternaria alternate* (Schuhegger *et al.* 2006).
B. AIMS OF THE STUDY

The quorum sensing (QS) system is a population density-dependent mechanism used by more than 50 species of Gram-negative bacteria. It is one of the main virulence regulators in *Erwinia carotovora* subsp. *carotovora* (*Ecc*). The overall aim of this work was to gain a deeper understanding of the QS regulatory mechanism and the QS regulon of the plant pathogen *Ecc*.

Studies focused on the following topics:
- Characterization of the N-acylhomoserine lactone (AHSL) synthase substrate specificity. Elucidation of the role of different types of AHSL molecules.
- Characterization of the QS regulators that respond to the AHSL and how this response mechanism functions and is transmitted to the target genes.
- Characterization of the QS regulon and identification of potential new QS target genes and their regulatory mechanism and function.
C. MATERIALS AND METHODS

Bacterial strains and plasmids used in this study are listed in Tables 3 and 4. The methods used in this study are described in detail in the indicated articles and are summarized in Table 5.

Table 3. Bacterial strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype/Relevant characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>recA thi pro hsdR M* RP4-2-Tc:MuKm Tn7 λ pir, TpR SmR</td>
<td>Miller and Mekalanos, 1988</td>
</tr>
<tr>
<td>S17-1 λ pir</td>
<td>endA1 hsdR17 supE44 thi-1 gyrA96 relA1 ΔlacU169 (φ80dlacΔM15)</td>
<td>Hanahan, 1983</td>
</tr>
<tr>
<td>DH5α</td>
<td>e14(McrA-) recA1 endA1 gyrA96 thi-1 hsdR17(xe- me-) supE44 relA1 Δ(lac-</td>
<td>Yanish-Perron et al. 1985</td>
</tr>
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<td>JM109</td>
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<td>Pirhonen et al. 1988</td>
</tr>
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<td>Pirhonen et al. 1991</td>
</tr>
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<td>Andersson (unpublished)</td>
</tr>
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<td>in SCC6005 background, KmR CmR</td>
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<td>Suicide vector, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
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### Table 5. Methods used in this study.

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<td>Assay for oxidative stress tolerance</td>
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<td>II, III</td>
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<td>Infection of potato with <em>Ecc</em></td>
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<td>Protein expression in <em>E. coli</em></td>
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<td>RNA isolation</td>
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<td>T4GT7 transduction</td>
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<td>Virulence test</td>
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<td>Western blot analysis</td>
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1. The acyl side chain of AHSLs defines the specificity of the communication system

1.1. Specificity of AHSL sensing in Ecc

Many Gram-negative bacteria use a population density-dependent cell-to-cell communication system called quorum sensing (QS) to control a wide variety of functions. The QS signaling molecule, N-acylhomoserine lactone (AHSL), acts as a “language” mediating the communication. These AHSLs are structurally diverse and differ in their acyl chain length, ranging from C4-C18, and in their substituent at the third carbon. This determines the signaling specificity in bacteria and enables the recognition and communication within bacterial species (Fuqua et al. 2002; Taga et al. 2003). To elucidate whether Erwinia carotovora subsp. carotovora (Ecc) strains differ in their capacity to sense AHSLs with different acyl side chain lengths, we studied the response of the expI mutant of Ecc strain SCC3193 (SCC3065) to different AHSLs. The AHSL synthase deficient expI mutant is cellulase negative and avirulent as such (Pirhonen et al. 1993), but the cellulase activity and thus the virulence can be restored by the addition of exogenous AHSLs.

The exogenous addition of AHSLs 3-oxo-C6-HSL and 3-oxo-C8-HSL showed that the cellulase production of the expI mutant could be restored by addition of less than 0.05 μM 3-oxo-C8-HSL, while 200 times more of 3-oxo-C6-HSL was needed (I, Figure 1). This suggested that Ecc strain SCC3193 is more specific for 3-oxo-C8-HSL. To analyze the AHSL profile we performed a liquid chromatography-mass spectrometry analysis of the growth culture supernatants of Ecc wild-type strains SCC3193 and SCC1, and the expI mutant (SCC3065). This showed that SCC3193 produced mainly 3-oxo-C8-HSL, SCC1 produced mainly 3-oxo-C6-HSL and the expI mutant produced no AHSL (I, Figure 2). To ensure that the type of AHSL produced is dependent on the AHSL synthase, we introduced expI\text{SCC3193} and expI\text{SCC1} in trans into the SCC3065 strain, thereafter the strains produced 3-oxo-C8-HSL and 3-oxo-C6-HSL, respectively. This suggested that the type of AHSL produced is solely dependent on the ExpI protein rather than on any other strain specific characteristic. But, why do two closely related Ecc strains use different QS signaling molecules? It might reflect a biological and/or ecological role for the choice of AHSL type and demonstrates the importance of bacteria to react when its own species members have reached a “quorum”. This may give an advantage in a competitive environment, where closely related bacterial species live in a similar niche.
1.2. Identification of critical residues in the AHS\textsubscript{L} synthase that determine acyl side chain length

Our results showed that the ExpI protein is responsible for producing AHSLs and determining the type of AHSL produced, specifying the “communication dialect” of the bacteria. We also showed that there is a difference in the bacterial sensing capacity for cognate versus non-cognate AHSLs, suggesting that each bacterial species understands best its own dialect. Therefore, the molecular basis of the ExpI protein was further investigated to understand why one \textit{Ecc} strain produces 3-oxo-C\textsubscript{6}-HSL and the other one 3-oxo-C\textsubscript{8}-HSL. We aligned closely related ExpI proteins, producing AHSLs with different chain lengths augmented with the crystal structure of the homologous EsaI of \textit{P. stewartii} (Watson \textit{et al.} 2002). The 3-oxo-C\textsubscript{6}-HSL producing EsaI of \textit{P. stewartii}, CarI of \textit{Ecc} GS101, ExpI of \textit{Ecc} of SCC1 and ExpI of \textit{Echr} 3937, and the 3-oxo-C\textsubscript{8}-HSL producing ExpI of \textit{Ecc} SCC3193 and ExpI of \textit{Ec} CFBP6272, were compared. A clear pattern of amino acid residues differentiating the two types of ExpI proteins, which produce either 3-oxo-C\textsubscript{6}-HSL or 3-oxo-C\textsubscript{8}-HSL was identified in the close proximity of the putative acyl substrate binding pocket determined in the crystal structure of EsaI (Watson \textit{et al.} 2002) (I, Figure 3). In the ExpI\textsubscript{SCC1} protein producing 3-oxo-C\textsubscript{6}-HSL, the identified residues were Leu-67, Phe-69, Leu-123 and Met-127, while the corresponding residues in the ExpI\textsubscript{SCC3193} protein producing 3-oxo-C\textsubscript{8}-HSL were Ser-67, Leu-69, Phe-123 and Thr-127.

To elucidate the role of the candidate residues in determining the acyl side chain length of an AHSL molecule we changed each of them in the ExpI\textsubscript{SCC1} to the corresponding residue in the ExpI\textsubscript{SCC3193}. In addition the residue 126 was mutated (Ser to Ala) due to its close proximity to the acyl substrate binding moiety, although the residue was not related to the pattern found in the comparison of the two ExpI proteins. The mutated \textit{expI} genes were introduced into the AHSL lacking strain (SCC3065) and thereafter the AHSL profile and the cellulase activity were analyzed (I, Figure 4). This analysis showed that residue 127 was the most critical for acyl side chain length specificity. The single change of M127T changed the former 3-oxo-C\textsubscript{6}-HSL producing ExpI\textsubscript{SCC1} to a 3-oxo-C\textsubscript{8}-HSL producing ExpI, however the amount of AHSL was drastically reduced. To confirm the importantance of residue 127, an opposite change of T127M in ExpI\textsubscript{SCC3193} was generated. This led to a similar effect with the former 3-oxo-C\textsubscript{8}-HSL producing ExpI\textsubscript{SCC3193} subsequently producing 3-oxo-C\textsubscript{6}-HSL in a similarly reduced amount. The single mutations L67S and F69L in ExpI\textsubscript{SCC1} did not affect the type of AHSL. However, these mutations reduced clearly the amount of AHSL produced. The single mutations L123F and S126A in ExpI\textsubscript{SCC1} did not alter the amount nor the type of AHSLs produced.
1.3. Mutations causing changes in bacterial communication

The M127T change in ExpI\textsubscript{SCC1} changed the communication language, but the AHSL amount generated (10 nM) was too low to restore the cellulase activity in the \textit{expI} mutant (SCC3065) background. This suggested that additional changes in the other residues of the putative substrate-binding pocket might be required for higher production level of AHSL. To test this hypothesis we systematically changed each of the identified amino acid residues in combination with the M127T change (I, Figure 4). The results showed that the minimum of amino acid changes required to alter the acyl chain length and to restore the AHSL production to a level that recovers the cellulase activity were F69L and M127T. The additional mutation of S126A further increased the synthesis level of AHSLs. To change the signal produced by ExpI\textsubscript{SCC1} and to fully restore the AHSL level, mutations in all five amino acids identified were required (L67S F69L L123F S126A M127T), resulting in even higher amounts of 3-oxo-C8-HSL compared to the wild-type ExpI\textsubscript{SCC3193}.

The obtained results are in accordance with the EsaI crystal structure, showing that in ExpI\textsubscript{SCC1} the Phe-69 and Met-127 residues are in close contact and seem to form the end of the C6-substrate binding pocket (I, Figure 6). The mutation at residue 127 from Met to the more hydrophilic amino acid Thr seems to have an influence on the contortion of the residue 127 and the change in the size of the acyl substrate binding pocket. Further the mutation at residue 69 from Phe to the smaller amino acid Leu seems to have an influence on the gap widening and thereby, allowing the larger C8 side chain to fit in (I, Figure 6). This theory is supported by a similar interpretation of structural studies with two LuxI-type proteins, EsaI and LasI, which generate 3-oxo-C6-HSL and 3-oxo-C12-HSL, respectively. These studies showed that in LasI, the acyl chain binding pocket is a tunnel which permits the end of the acyl chain to protrude, whereas in EsaI the acyl chain binding pocket is closed. The residues in EsaI that occlude the pocket are larger than those at the same position in LasI, thus limiting the acyl chain size to a C6 acyl chain (Watson \textit{et al.} 2002; Gould \textit{et al}. 2004). The production of 3-oxo-C6-HSL is almost completely abolished in the mutant F69L M127T. This may be because the amino acid changes lead to a reduced overall effectiveness of the catalytic process with C6-substrates.

To demonstrate that the decreased AHSL amount synthesized by the M127T mutant was not due to protein instability nor differences in the copy number we constructed His\textsubscript{6} -tagged variants of ExpI\textsubscript{SCC1} wild-type and the mutants M127T and F69L M127T (I, Figure 5). The Western blot analysis showed that the amount of ExpI protein produced was similar in all cases though the AHSL levels varied markedly. This indicated that the differences in AHSL levels are due to divergence in the ExpI activity.

Of note is that in this study only two amino acid changes in one protein were shown to be enough to change the communication language, an event that has a profound effect on the virulence of the bacteria. Furthermore, these findings suggest that bacteria have evolved a specific system, in which, an AHSL counterpart is used to distinguish between the specific dialects.
2. ExpR1 and ExpR2 control virulence and quorum sensing specificity

The plant pathogenic *Ecc* use mainly two different QS signaling molecules, 3-oxo-C6-HSL or 3-oxo-C8-HSL, for communication (Barnard and Salmon, 2007). The bacteria are specific for their own dialect and respond only to the cognate AHSL molecule at physiological concentrations (I). But how do bacteria listen and respond to the communication?

2.1. ExpR1 and ExpR2 act as negative regulators of virulence

In order to elucidate the role of the AHSL counterpart and its ability to listen to the communication by sensing different types of signaling molecules, we characterized the specificity of the LuxR-type protein, the QS regulator ExpR (Andersson *et al.* 2000) in *Ecc* strain SCC3193. For this purpose we tested the ability of the cognate 3-oxo-C8-HSL and the non-cognate 3-oxo-C6-HSL to restore the cellulase activity in an *expI* mutant (SCC3065) and in an *expI expR* double mutant (SCC6005). Interestingly, the cellulase activity of the *expI expR* double mutant could be restored by the addition of physiological levels of cognate as well as non-cognate AHSLs (II, Figure 1), while cellulase activity of the *expI* mutant could only be restored by the addition of cognate AHSL, as earlier described (I, Figure 1). We also tested the specificity of the *expI expR* double mutant for other AHSLs and indeed the cellulase activity was restored with exogenously added AHSLs ranging from C6-HSL to 3-oxo-C10-HSL (II, Figure 3). However, the *expI expR* mutant was not able to sense AHSLs with longer acyl chains (C12 and C14), which may depend on the lack of a reasonable transport system into the cell for long chain AHSLs (Fuqua *et al.* 2001).

The absence of the *expR* gene had a profound effect on the AHSL sensing specificity. To determine whether the sensing specificity was due to the presence of the ExpR protein the *expR* gene was expressed in trans in the *expI expR* double mutant. The results showed that ExpR requires the cognate 3-oxo-C8-HSL for restoration of the cellulase activity, indicating that ExpR determines the communication language used. In addition, these results suggested that there is also another mechanism, which is able to sense “listen to” a broader range of QS signaling molecules.

From the sequenced genome of *Erwinia carotovora* subsp. *atroseptica* (*Eca*) strain SCRI1043, two *expR* genes have been identified (Bell *et al.* 2004). One chromosomally linked to the *expI* gene as in *Ecc* strain SCC3193 and the other one existing separately. Using PCR we were able to identify the existence of a second *expR* gene in the *Ecc* strain SCC3193 (designated *expR2*, with the previously identified *expR* renamed *expR1*). Structural comparison of the newly found ExpR2 and the LuxR-type proteins of *Erwinia carotovora* subsp. and *P. stewartii* showed that the DNA-binding domain is highly conserved, while the AHSL-binding domain showed more sequence variability (II, Figure 2). This supported our observation that the newly identified ExpR2 might have a different sensing specificity compared to the earlier identified ExpR1.
Previously, the ExpR1 of Ecc strain SCC3193 was suggested to have a role as a repressor. An expR1 mutant was shown to have a slightly increased maceration capacity and AHSL production compared to the wild-type (Andersson et al. 2000). To further explore the role of the two ExpR proteins the expI expR1, expI expR2 and expI expR1 expR2 mutants of Ecc strain SCC3193 were characterized for their role in the regulation of PCWDEs. The presence of either expR1 or expR2 in the expI mutant background resulted in a cellulase negative phenotype. Interestingly, the simultaneous inactivation of both expR genes in the expI mutant background fully restored the cellulase activity (II, Figure 3). This data indicates that both ExpR1 and ExpR2 act as negative regulators of PCWDE production and hence influence the virulence of Ecc. Similar findings were also presented for the LuxR-type proteins VirR of Eca strain SCR1043 and ExpR2 of Ecc strain Ecc71 (Burr et al. 2005; Cui et al. 2006). Contrary to our findings, mutations in virReca and expR2Ecc71 alone were able to restore the cellulase activity of the corresponding expI mutants. However the ExpR1 homolog was not alone sufficient to restore the cellulase activity in these strains (Burr et al. 2005; Cui et al. 2006). This variability in the regulatory mechanisms between different strains of Erwinia carotovora subsp. suggests that all QS systems, even in closely related strains, act slightly differently and are tailor-made for specific requirements in the environment bacteria are living in.

It is intriguing that the expI expR1 expR2 triple mutant lacking the whole QS system was able to grow and macerate plant tissue as well as the wild-type in laboratory conditions (II, Figure 8). This raises the question of the role of the whole QS system. One suggestion is that QS is vital only in the natural habitat, where cell densities and the composition of bacterial populations fluctuate in response to environmental cues. It has also been suggested that QS has a role in transition from biotrophy to necrotrophy. This is supported by recent findings in Eca showing that in addition to PCWDEs QS regulates the entire T3SS, its effectors and the synthesis of coronafacic acid, which all have a role in the suppression of plant defense (Toth et al. 2005; Liu et al. 2008).

2.2. ExpR1 and ExpR2 bind AHSL and determine the AHSL sensing specificity of Ecc

Most LuxR-type proteins known today are especially sensitive for their cognate AHSL, with the acyl side chain being the major specificity determinant (Welch et al. 2000; 2005; I). To analyze the specificity of ExpR1 and ExpR2, the expI expR1 and the expI expR2 mutants were investigated for their ability to restore cellulase activity in response to different AHSLs. The expI expR2 mutant responded only to the cognate AHSL 3-oxo-C8-HSL at the physiological level suggesting that ExpR1 is responsible for the QS specificity for the cognate AHSL. On the other hand the expI expR1 mutant was able to restore the cellulase activity in response to both cognate and non-cognate AHSLs, suggesting that the ExpR2 protein has broader sensing capacity (II, Figure 3). Recently, similar kinds of results have been obtained with the Ecc strain Ecc71, where ExpR2 was shown to activate the target gene expression in the presence of both 3-oxo-C6-HSL and 3-oxo-C8-HSL (Cui et al. 2006).
To demonstrate that the QS regulators ExpR1 and ExpR2 are responsible for specific binding of the AHSL molecules, we measured the amount of exogenous AHSL bound to cell extracts using a bioluminescence assay. In the \textit{expI} mutant, conferring both ExpR1 and ExpR2 proteins, both 3-oxo-C6-HSL and 3-oxo-C8-HSL were bound. The 3-oxo-C8-HSL was bound even more effectively, which was most likely due to the presence of both ExpR1 and ExpR2 proteins, both able to bind the cognate AHSL (II, Figure 5). The \textit{expI expR2} mutant bound only to the cognate AHSL 3-oxo-C8-HSL, while the \textit{expI expR1} mutant was able to bind both 3-oxo-C6-HSL and 3-oxo-C8-HSL in almost equal amounts. As expected in the \textit{expI expR1 expR2} triple mutant no AHSL was bound to the cell extract. These results support the role of ExpR1 as a specific and ExpR2 as an unspecific AHSL receptor. Furthermore these results suggested that ExpR1 and ExpR2 act in synergy in binding the cognate AHSL.

2.3. ExpR1 and ExpR2 control RsmA, a global negative regulator of virulence

The ExpR1 and ExpR2 proteins control negatively the production of PCWDEs. But, is this regulation direct or mediated by some other factor? In a recent study, it was shown that in \textit{Ecc} the expression of \textit{rsmA}, the global negative regulator of virulence, was affected by the presence of AHSL. The level of \textit{rsmA} mRNA was highly increased in the \textit{expI} mutant compared to the wild-type (Cui \textit{et al.} 1995; Mäe \textit{et al.} 2001). This prompted us to investigate in the role of ExpR1 and ExpR2 in the expression of \textit{rsmA}.

For this purpose, we tested the activity of an \textit{rsmA-gusA} promoter fusion in different mutant backgrounds (II, Figure 5). The \textit{expI} mutant, grown either without cognate AHSL or with the addition of the non-cognate 3-oxo-C6-HSL, showed increased activity of the \textit{rsmA-gusA} promoter fusion, while this was suppressed with 3-oxo-C8-HSL. The \textit{expI expR2} double mutant acted in the same manner. We suggest this is due to the presence of the ExpR1 protein, which activates the \textit{rsmA} expression in an AHSL free state. The addition of 3-oxo-C8-HSL leads to binding of the autoinducer to the ExpR1 protein and suppresses the activation of \textit{rsmA} expression. On the other hand, in the \textit{expI expR1} mutant, the \textit{rsmA-gusA} promoter fusion was only activated in the absence of AHSLs, since the presence of any AHSL bound to the ExpR2 releases ExpR2 from the \textit{rsmA-gusA} promoter fusion activation. In the \textit{expI expR1 expR2} triple mutant, the activation of \textit{rsmA} was suppressed in all cases due to the lack of an activating ExpR protein (II, Figure 5). These results showed that the QS responsive virulence regulation is indeed mediated via RsmA. In conclusion, in the absence of AHSL, the ExpR1 and ExpR2 proteins could both, either together or separately, activate the expression of \textit{rsmA}. The addition of AHSLs suppressed the activation of \textit{rsmA} transcription. This suppression acted in accordance with the ligand binding specificity identified for the ExpR1 and ExpR2 proteins. Furthermore, the \textit{rsmA-gusA} promoter fusion results together with the AHSL binding results showed that ExpR1 and ExpR2 can act independently, but also in synergy, though not necessarily physically interacting. These results are supported by the recent findings in Ecc71 showing that ExpR\textit{71} binds to the promoter of \textit{rsmA}. The activation
of the rsmA transcription could be prevented by the addition of 3-oxo-C6-HSL (Chatterjee et al. 2005; Cui et al. 2005). In addition, it was shown that the rsmA promoter of the Ecc strain Ecc71 contain two ExpR binding sites. A stronger regulatory role was proposed for ExpR2 that bind to the promoter region, upstream of the ExpR1-binding site (Cui et al. 2006). In contrast, our rsmA-gusA promoter fusion results, suggest a slightly stronger activating role for the ExpR1 protein.

A similar type of mechanism as shown here for ExpR1 and ExpR2 has been proposed for EsaR of P. stewartii. In this case, EsaR binds to its target gene promoter in the absence of AHSL and represses the transcription, while this repression is released by the addition of the cognate AHSL (Minogue et al. 2005). The model is the opposite in LuxR-type activators, such as CarR, LuxR and TraR that dimerize/multimerize after binding AHSL and subsequently bind to the target gene and activate its transcription (Qin et al. 2000; Welch et al. 2000; Urbanowski et al. 2004).

2.4. ExpR1 changes the communication language

We hypothesize that the specificity of the communication dialect is largely dependent on the QS regulator ExpR1 and can be altered by exchanging the ExpR1 protein. To further elucidate this hypothesis expR1 of Ecc strain SCC1, a 3-oxo-C6-HSL producing strain (I), was inserted to the expl expR1 expR2 triple mutant and the cellulase activity was analyzed. This, indeed, converted the former 3-oxo-C8-HSL responding strain to a 3-oxo-C6-HSL responding strain (II, Figure 7). Also the introduction of expR1SCC1 to the expl expR1 mutant converted the former 3-oxo-C8-HSL responding strain to a 3-oxo-C6-HSL responding strain. However, the introduction of the foreign expR1SCC1 to the expl mutant and the expl expR2 double mutant resulted in a cellulase negative phenotype, independent of AHSL type added. This could be explained by the simultaneous presence of two ExpR1-type regulators that are specific for different AHSLs. The results suggest that the ExpR1 protein has a crucial role, which can not be adapted by ExpR2, in determining the AHSL sensing specificity of the bacteria. The presence of two ExpR proteins with different binding capacity is of high interest. One advantage could be the ability to sense neighbouring bacteria by responding to different types of AHSL. This would allow eavesdropping on possible competitors and activation of the pathogen’s defense responses (Lazdunski et al. 2004; Waters and Bassler, 2005). Another advantage could be cooperation with other bacteria to overwhelm the plant host, which would enhance the survival rate in a crowded niche. To better understand the role of the QS system it would be of importance to further elucidate the QS regulon.
3. Identification of new quorum sensing targets in Ecc

A transcriptome analysis performed on the opportunistic human pathogen *P. aeruginosa* grown in minimal medium showed that QS is a global regulator affecting the expression of several hundreds of genes (Wagner et al. 2003). Also, in *Ecc* and *Eca*, both proteome and transcriptome analysis have been conducted in order to elucidate the role of QS in the *Erwinia*-host interaction (Corbett et al. 2005; Pemberton et al. 2005; Liu et al. 2008). These results showed that also in *Erwinia carotovora* subsp., QS is a global regulator, which controls the expression of several target genes, in addition to the PCWDEs and *hrpN* identified earlier. However, it remains to be clarified whether these genes are controlled directly or indirectly by the QS regulators. The global negative regulator RsmA has been identified as a direct target of the QS regulators ExpR1 and ExpR2 in *Ecc* (Cui et al. 2005; Cui et al. 2006; II). To further understand the role of the two QS regulators and to identify new targets of the QS system in *Ecc* strain SCC3193 a transposon mutagenesis screen was performed.

3.1. Identification of a plant ferredoxin-like protein and the regulator Hor as quorum sensing targets

In this study random transposon mutagenesis was employed by introducing a mini-Tn5Cm::gusA transposon into the *expI* mutant strain SCC3065 to identify novel QS targets (Pirhonen et al. 1993). The transposon mutants were screened for their GUS activity in the absence and presence of 1 µM AHSL. This screen revealed two new genes, *ferE* and *hor*, whose expression was clearly responsive to AHSL. The *ferE* response to AHSL was even stronger than the effect of AHSL on the *hor* expression, which showed a somewhat higher basal level of GUS activity compared to *ferE* (III, Figure 1).

The *ferE* encodes for a putative ferredoxin protein, which is most similar to cyanobacterial and plant ferredoxins, but with only a limited similarity to ferredoxins from other bacterial genomes (III, Figure 3). The genome of *Eca* strain SCRI1043 possesses at least 10 genes encoding different kinds of ferredoxins (GenBank|BX950851), but the gene products are clearly distinct from the plant ferredoxin-like protein characterized here. The putative FerE protein of *Ecc* strain SCC3193 has high amino acid sequence similarity to Ferredoxin I (FedI, PetF) (64% identity and 76% similarity) of cyanobacteria (Floss et al. 1997) and to plant ferredoxins of the Fd2-type (FedA) (56% identity and 76% similarity) of *Arabidopsis thaliana* (Hanke et al. 2003). Ferredoxins are iron-sulphur proteins that mediate electron transfer in a range of metabolic reactions, such as nitrogen and sulphur assimilation, sulphite reduction, amino acid and fatty acid metabolism and redox regulation, as well as mediation of electron transfer from the photosystem I to the ferredoxin NADP reductase (Hanke et al. 2003; Mazouni et al. 2003).
We suggest that the ferE gene of Ecc strain SCC3193 has been acquired through horizontal gene transfer (HGT). This is supported by the sequence comparison analysis where the highest amino acid sequence identity to any other known bacterial protein, with an exception of proteins of cyanobacteria and Actinobacillus succinogenes, was found to be less than 30%, which was detected for the phenylacacetate-CoA oxygenase/reductase of E. coli B. Also the G+C content of the ferE gene is lower (37%) than the overall G+C content (50.9%) of the Eca genome, a close relative to Ecc (Bell et al. 2004). It is even closer to the overall G+C content (43%) of the A. thaliana genome (Kuhl et al. 2005). It has been estimated that 6 to 21% of the genes of plant pathogenic bacteria might be acquired by HGT. In accordance, analysis of the Eca genome indicates a substantial percentage of genes acquired by HGT (Toth et al. 2006). While most HGT derived genes seem to originate from other bacteria, a smaller but still significant number of genes (around 1%), particularly in plant and animal pathogens and symbionts appear to have been acquired from eukaryotes (Koonin et al. 2001; Brown, 2003).

The other new QS target identified here, the hor gene, encodes for a putative DNA-binding Hor protein (homologous of rap), member of the MarR/SlyA-transcriptional regulator family known to control antimicrobial activities (Thomson et al. 1997). It is closely homologous to other Hor proteins in Erwinia carotovora subsp. and in Serratia (III, Figure 2). In the opportunistic pathogen Serratia, the Hor homolog Rap is controlled by QS and regulates the biosynthesis of the antibiotic carbapenem in a QS-dependent manner (Slater et al. 2003; Fineran et al. 2005). However, hor was shown not to be controlled by QS in the other Ecc strains investigated (McGowan et al. 2005). Therefore it is especially interesting to find hor as a QS controlled gene in the Ecc strain SCC3193 investigated here. Nevertheless, as in Serratia, in certain strains of Ecc Hor controls the expression of genes for carbapenem biosynthesis, which again are directly regulated by the QS regulator CarR (Holden et al. 1998; Thomson et al. 1997; 2000). The Ecc strain SCC3193 has been shown to lack the carbapenem synthetising genes (Holden et al. 1998). Thus it remains to be clarified, what is the target of the putative regulator Hor of Ecc strain SCC3193 and whether it might have a role in controlling some other antimicrobial processes.

3.2. ExpR1 and ExpR2 control the expression of ferE and hor via RsmA

The QS regulators ExpR1 and ExpR2 have been shown to regulate their target genes in an AHSL dependent and specific manner (Cui et al. 2006; II). To explore how the QS regulated expression of ferE and hor is executed, we employed a Northern blot analysis (III, Figure 4). As expected, in the expI mutant the expression of both ferE and hor was downregulated in the absence of AHSLs, but it could be upregulated by addition of the cognate AHSL. Then again in the expI expR1 mutant the expression of both target genes was activated with the addition of either 3-oxo-C6-HSL or 3-oxo-C8-HSL, while in the expI expR2 mutant the expression of ferE and hor could be activated only by the addition of the cognate AHSL. In the expI expR1 expR2 triple mutant the hor and ferE expression was upregulated under all conditions. Taken
together, these results suggested that in the absence of AHSLs ExpR1 and ExpR2 act as negative regulators of ferE and hor, while this could be suppressed by the addition of AHSL. In other words, the gene expression of ferE and hor was shown to be regulated by ExpR1 and ExpR2 in an AHSL dependent and specific way.

To further investigate whether the ExpR1 and ExpR2 mediated regulation of ferE and hor is direct or indirect, we explored the role of the global negative regulator RsmA in controlling the expression. For this purpose an expI rsmA mutant strain was used (Andersson, unpublished). Interestingly, in this mutant the expression of ferE and hor was upregulated both in the presence and absence of AHSLs and thus independent of AHSL (III, Figure 5). This suggested that the QS control of the ferE and hor expression is also mediated via RsmA.

These findings extend the list of known RsmA target genes. From previous studies, except for the PCWDEs, RsmA is known to regulate the expression of hrpN in Ecc strain Ecc71 (Cui et al. 1996) and several virulence-related genes in e.g. P. aeruginosa, including rhamnolipid biosynthetic operons, hydrogen cyanide and the AHSL synthase gene (Pessi et al. 2001; Heurlier et al. 2004). As our results enlarge the RsmA mediated QS regulon, it triggers the question whether all QS control in Ecc is mediated by RsmA or is there also an RsmA independent QS regulatory mechanism, regulated directly by ExpR1 and/or ExpR2?

3.3. Contribution of Hor and FerE to virulence

Earlier reports have shown that Hor has a role in virulence regulation (Holden et al. 1998). This and the lack of carbapenem synthesizing genes in Ecc strain SCC3193 prompted us to investigate how the inactivation of hor affects the virulence of Ecc strain SCC3193. Potato tuber slices (cultivar Van Gogh) and A. thaliana Col-0 plants were used as model organisms in infection studies. In both plant species the plant maceration was clearly reduced in the hor mutant compared to the wild-type (III, Figure 6). This was in accordance with the reduced production of PCWDEs in a hor mutant as assessed by indicator plates. To further investigate the role of Hor in virulence we measured the in planta growth of the hor mutant in A. thaliana Col-0. This showed a slightly reduced growth compared to the wild-type strain.

As a ferE mutant has not been characterized earlier in heterotrophic bacteria, we were interested to study whether it has also an effect on the virulence. However, the ferE mutant showed wild-type level of plant maceration and PCWDE production. Nevertheless, as the hor mutant, also the ferE mutant showed slightly reduced growth in A. thaliana Col-0. This indicated that both Hor and FerE could contribute to the overall fitness of bacteria during infection.
3.4. Quorum sensing regulate oxidative stress tolerance

The FerE of Ecc strain SCC3193 contains a conserved putative iron-sulfur binding domain 2Fe-2S. In E. coli and E. chrysanthemi proteins with iron-sulphur clusters have often been shown to have an effect on bacterial tolerance to oxidative stress (Koo et al. 2003; Ollagnier-de Choudens et al. 2003). This prompted us to assess the role of FerE in oxidative stress tolerance. The ferE mutant strain was exposed to 20, 30 and 40 mM H₂O₂ and tested for its susceptibility. The mutant strain showed clear concentration-dependent reduction in oxidative stress tolerance (III, Figure 7). Previously QS has been shown to control oxidative stress in P. aeruginosa (Hasset et al. 1999; Quinones et al. 2005) and in Burkholderia pseudomallei (Lumjiakatse et al. 2006). Since the role of QS in oxidative stress tolerance of Ecc has not been characterized earlier we tested the effect of H₂O₂ on the expI mutant. These results demonstrated that the expI mutant was clearly sensitive to H₂O₂ with 40 mM H₂O₂ being lethal for the mutant. This suggested that oxidative stress tolerance is QS-dependent. Consistently, this deficiency could be complemented with the addition of 1 µM 3-oxo-C8-HSL (III, Figure 7). To further analyze the role of FerE, the ferE gene was expressed in trans both in the ferE mutant and in the expI mutant. The reduced oxidative stress tolerance of the ferE mutant was complemented by the addition of the ferE gene in trans. The expression of ferE in trans in the expI mutant partially restored the oxidative stress tolerance of the expI mutant, indicating that FerE contributes to the QS controlled oxidative stress tolerance. Our results support the hypothesis that also in Ecc, QS is one of the main regulators of oxidative stress. Since oxidative burst plays a central role in many host-pathogen interactions the role of a population density -dependent mechanism as a key regulator of oxidative stress tolerance is legitimate.
E. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Quorum sensing (QS) is a central regulator of virulence and essential for a successful infection in the plant pathogen *Erwinia carotovora* subsp. *carotovora* (*Ecc*). Together with QS several other virulence regulators of *Ecc*, form a complex and hierarchical regulatory network that is responsible for a coordinated production of the PCWDEs, the main virulence determinants. In this study we were able to further deepen the understanding of the QS system; to recognize the features of the signaling molecule specificity, how the QS signal is received and how the information is transmitted to the target genes and to widen the knowledge of the QS regulon of *Ecc*.

The *Ecc* QS system has been under close investigation for more than 15 years and for long the PCWDEs and the β-lactam antibiotic carbapenem were considered as the major targets. However, recently with the sequencing of the *Eca* genome, the width of the QS regulon and its position as a master regulon in the hierarchical regulatory network has become clearer (Liu *et al.* 2008). The extensiveness of the QS regulon was also supported by the results in this thesis study. We were able to identify two new targets with widely different functions. One target being Hor, a DNA-binding regulator that was demonstrated to control the PCWDE production. Hor has earlier been shown to regulate the biosynthesis of carbapenem, which does not exist in the *Ecc* strain used in our studies. A possible role in some other antimicrobial process can not be ruled out and remains to be clarified. The other target identified in our study was FerE, a putative plant-like ferredoxin. The *ferE* mutant had lowered oxidative stress tolerance and interestingly an *expI* mutant was even more susceptible, being completely unable to grow, when encountered with 40 mM H$_2$O$_2$. This suggested that QS is a key regulator of oxidative stress tolerance, which is one of the major pathogen induced plant defense responses.

Moreover our studies revealed that both the QS regulators ExpR1 and ExpR2 act as negative regulators of PCWDE production at low population density and this repression is relieved by interaction with AHSLs at high population density. Furthermore we showed that a simultaneous inactivation of both ExpR proteins was needed for the suppression of the avirulent phenotype of the *expI* mutant. Surprisingly the *expI expR1 expR2* triple mutant lacking the whole QS system was able to grow and macerate plant tissues as well as the wild-type in laboratory conditions. This triggers the question about the role of QS in *Ecc*. One hypothesis is that QS has significant role also at early stages of the infection process by delaying the plant defense response, until bacteria reach a population density high enough for production of PCWDEs and subsequently a successful infection (Pirhonen *et al.* 1993; Toth *et al.* 2005). The newly identified QS targets are likely to be active during different phases of the infection process and further underline the wide impact QS might have.

In addition, we demonstrated that the control of ExpR1 and ExpR2 on target genes was mediated by the global negative regulator RsmA. The RsmA is a key component of several regulatory cascades of *Ecc* and is itself subject to control by several regulatory factors (Figure 5). It remains to be clarified whether all QS control is mediated by RsmA, or
alternatively, do the QS regulators also have other direct or indirect targets. By assuming that not all ExpR regulation is mediated by RsmA, then the question is whether ExpR1 and ExpR2 regulate separate targets or do they always cooperate by regulating the same targets and using the double ExpR appearance as a fine-tuning mechanism. This topic is even more intriguing as we demonstrated that the two ExpR proteins have different binding specificity, with ExpR1 binding cognate AHSL and ExpR2 binding both cognate and non-cognate AHSLs. This raises the question whether ExpR2, has unique target sites, activated by the binding of non-cognate AHSLs and possibly involved in interactions with other bacterial species.

As it was shown that ExpR2 is able to respond to a variety of AHSLs, it was equally interesting to find that two closely related Ecc strains synthesize different AHSLs, 3-oxo-C6-HSL and 3-oxo-C8-HSL. Furthermore we found that this was only due to two amino acid substitutions (F69L M127T) changing an AHSL synthase producing 3-oxo-C6-HSL to produce 3-oxo-C8-HSL. But, why do two closely related Ecc strains use different QS signaling molecules and sensing systems? One hypothesis is that it is of importance for bacteria to be able to sense and react when its own species members have reached a “quorum”. This would help in a competitive environment, where closely related bacterial species live in the same niche. Additionally the existence of the unspecific QS regulator ExpR2 could allow eavesdropping on possible competitors and activation of bacterial defense responses. Alternatively this could allow cooperation with other bacteria to overwhelm the plant host enhancing the survival rate in a crowded niche.

One of the long-term objectives of this research topic is to find better means how to defend plants from bacterial diseases. As QS is a central regulator of virulence, it would be meaningful to find a strategy that impedes the QS system, which could be useful for plant defense. However, many questions still need to be assessed, before this can be achieved. With the increase in available genomic sequence data, one alternative way to approach some of these matters is with proteomic and transcriptomic analysis between different mutant strains. Such studies will be facilitated by the recent sequencing of the genomes of both Ecc strain SCC3193 and SCC1 by our laboratory. For example an experiment comparing the mutants expl, expl expR1, expl expR2 and expl expR1 expR2 grown in the absence and presence of both cognate and non-cognate AHSL molecules would partly solve some of the questionmarks illustrated in Figure 5.
To summarize, on the bases of our studies, some of the next questions to be answered are:

- Is all QS control mediated by RsmA or does ExpR1 and ExpR2 have also other targets?
- Does ExpR1 and ExpR2 possess own, separate targets or do they always act in concert on common targets?
- Do ExpR1 and ExpR2 interact physically?
- Does ExpR2 have unique targets responding only to non-cognate AHSLs?
- Why do two closely related Ecc strain use different AHSL signals?
- What is the role of the QS system, as removal of the whole system, results in wild-type virulence capacity under standard laboratory conditions?
F. ACKNOWLEDGEMENTS

This work was carried out at the Department of Biological and Environmental Sciences, Division of Genetics, University of Helsinki, in the Viikki Biocenter. Funding was provided by Academy of Finland and Helsinki Graduate School in Biotechnology and Molecular Biology.

I am grateful to my supervisors, Professor Tapio Palva and Docent Günter Brader. I thank Tapio for giving me the opportunity to work in his group with the fascinating world of Erwinia and quorum sensing. Tapio’s constructive feedback and visions have been invaluable. I also thank Tapio for his flexibility in finalizing this thesis. I am indebted to Günter for his optimistic and positively critical guidance and for many long and delightful scientific discussions.

Docent Benita Westerlund-Wiklund and Professor Jari Valkonen are especially thanked for critically reviewing this thesis, which remarkably improved it. I am thankful to my follow-up group members Minna Pirhonen and Teemu Teeri for their support.

Warm thanks are owned to the former and present members of Tapio Palva’s, Pekka Heino’s and Hannu Saarilahti’s groups for collaboration, good companionship and joyful lunch breaks. Special thanks are to Elina Helenius, Heidi Hyytiäinen, Tarja Kariola, Hanne Mikkonen, Tiina Palomäki and Anne Tuikkala with whom I have shared many good moments during these years. I am also thankful to all plant-pathogen interaction people, especially Karen Sims-Huopaniemi and Jing Li. Leila Miettinen is thanked for valuable help. I am particularly indebted to Heidi Hyytiäinen for introducing me to the secrets of Erwinia, and to Heidi Harjunpää and Gudrun Koch for excellent and enthusiastic assistance, which largely contributed to this work. Pekka Heino and Hannu Saarilahti are acknowledged for valuable advices during the years. I am also grateful to Arja Välimäki and Arja Ikävalko for generous help in different matters.

Innomedica Ltd, my present employee, I wish to acknowledge for their flexibility and positive attitude.

I am deeply grateful to Marie Johansson and Minna Mäki for invaluable help especially in the last stages of this work. Also I am warmly thankful to Marie, for being a great friend and for endless joyful discussions regarding all matters in life and to Minna, for her optimism, support and excellent sparring on the running field.

I also wish to thank my friends Jenny Harmsen and Jenny Carlberg for their care and sincere friendship throughout the years. Nina Järvenpää, Minna Mäki and Jaana Niittymäki are acknowledged for the refreshing WA-meetings. I am also indebted to my relatives and my other friends outside the science community for keeping my view of life open and curios.

I like to sincerely thank my parents in law, Kaisu and Anders, for always being helpful and for wonderful childcare.
My heartfelt thanks go to my mother Airi, my late father Bo and my brother Björn and his family, for their love and encouragement and for giving me a creative and inspiring atmosphere to grow up in.

Finally, my sincerest gratitude belongs to my dear husband, my best friend Magnus, for all the fun, help and patience, and to our dear son, Bernard, for all the joy he brings each day.

Helsinki, May 2009

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G. REFERENCES


Guard-Petter, J. 1998. Variants of smooth Salmonella enterica serovar enteritidis that grow to higher cell density than the wild-type are more virulent. Appl Environ Microbiol. 64:2166-2172.


**dieffenbachiae** sp. nov. and *Dickeya zeae* sp. nov. Int J Syst Evol Microbiol. 55:1415-27.


