QUORUM SENSING IN THE PLANT PATHOGEN *ERWINIA CAROTOVORA* SUBSP. *CAROTOVORA*

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Academic dissertation

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Dedicated to my father for everlasting encouragement.

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications which are referred to by their Roman numerals in the text.

I Brader, G., Sjöblom, S., Hyytiäinen, H., Sims-Huopaniemi, K. and Palva, E.T. 2005. Altering substrate chain length specificity of an acylhomoserine lactone synthase in bacterial communication. J Biol Chem. 280:10403-10409.

II Sjöblom, S., Brader, G., Koch, G. and Palva, E.T. 2006. Cooperation of two distinct ExpR regulators controls quorum sensing specificity and virulence in the plant pathogen *Erwinia carotovora*. Mol Microbiol. 60:1474-1489.

III Sjöblom, S., Harjunpää, H., Brader, G. and Palva, E.T. 2008. A novel plant ferredoxin-like protein and the regulator Hor are quorum sensing targets in the plant pathogen *Erwinia carotovora.* Mol Plant-Microbe Interact. 21:967-978.

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 $ExpI_{SCC1}$ mutations M127T and F69L are sufficient to change the *N*-3-oxohexanoyl-L-homoserine lactone producing $ExpI_{SCC1}$ to an *N*-3-oxooctanoyl-L-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 iaonatha*), 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 *Erwinia*s 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-l 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*.

Enzyme		**Strain**	**Reference**
Cellulases	CelA	LY34	Park *et al.* 1997
	CelB	LY34	Park *et al.* 1997
	CelS	SCC3193	Saarilahti *et al.* 1990a
	CelVI	SCC3193	Mäe *et al.* 1995
	CelV	SCRI193	Cooper and Salmond, 1993
Pectate lyases	PelA	EC	Lei *et al.* 1988
	PelB	EC	Lei *et al.* 1988
	Pel153	EC153	Trollinger *et al.* 1989
	Pel-1	ECC71	Chatterjee *et al.* 1995a
	Pel-3	ECC71	Liu *et al.* 1994
	PelII	ER	Yoshida *et al.* 1992
	PelIII	ER	Yoshida *et al.* 1991
	PelI	ER	Ito *et al.* 1988
	PelX	ER	Ito *et al.* 1988
	PelB	SCC3193	Heikinheimo *et al.* 1995
	PelB	SCRI193	Hinton *et al.* 1989
	PelC	SCRI193	Hinton *et al.* 1989
Pectin lyases	PnlA	ECC71	Chatterjee *et al.* 1991; McEvoy *et al.* 1990
	Pnl	ER	Ohnishi *et al.* 1991
Polygalacturonases	Peh	EC	Lei *et al.* 1992
	Peh-1	ECC71	Liu *et al.* 1994
	PehA	SCC3193	Saarilahti *et al.* 1990b
	Peh	SCRI193	Hinton *et al.* 1990
Proteases	Prt1	EC14	Kyöstiö *et al.* 1991
	PrtW	SCC3193	Marits *et al.* 1999

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 *Xanthomonas campestris*) and Nep1-like proteins (a new family of well conserved proteins that induce an HR-like response) (Pemberton *et al*. 2004; 2005; Mattinen *et al*. 2004; Corbett *et al*. 2005; Toth *et al*. 2003; Toth and Birch, 2005). *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 *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 *out* cluster (Johnson *et al*. 2006). The importance of the T2SS in *Ecc* is highlighted in a study where mutations affecting the T2SS resulted in an avirulent phenotype (Pirhonen *et al*. 1991). The type III secretion system (T3SS) is described below.

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

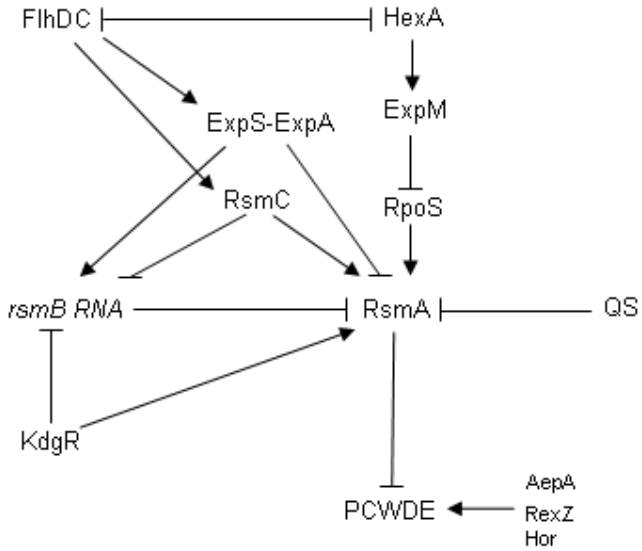


Figure 1. Schematic representation of the complex regulatory network involved in controlling PCWDE production in *E. carotovora* subsp. *carotovora*. Arrowheads indicate positive regulation and flattened ends denote negative regulation. Adapted and modified from Whitehead *et al*. 2002.

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). KdgR binds directly to three conserved KdgR-boxes in *rsmB*, preventing interaction of *rsmB* with RsmA (Nasser *et al*. 1994; Liu *et al*. 1999). 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* (Hugovieux-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 Pnl 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 down stream 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 *Eca*, 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).

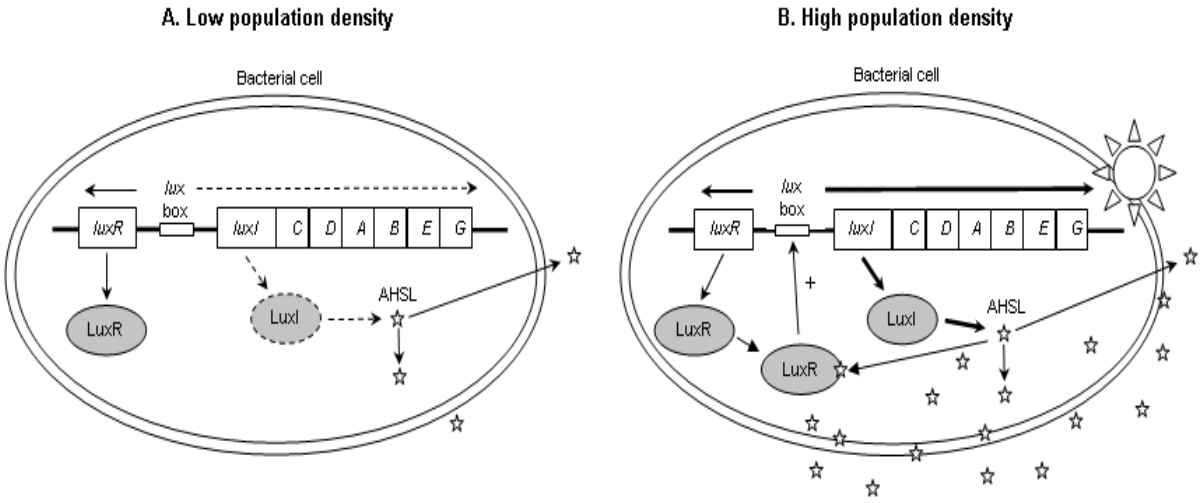


Figure 2. The basic QS system demonstrated by the regulation of bioluminescence in *V. fischeri.* **A.** At low population density, expression of the genes for bioluminescence (*luxICDABEG*) is weak and insufficient for light emission due to low levels of AHSL. **B.** At high population density, a critical 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).

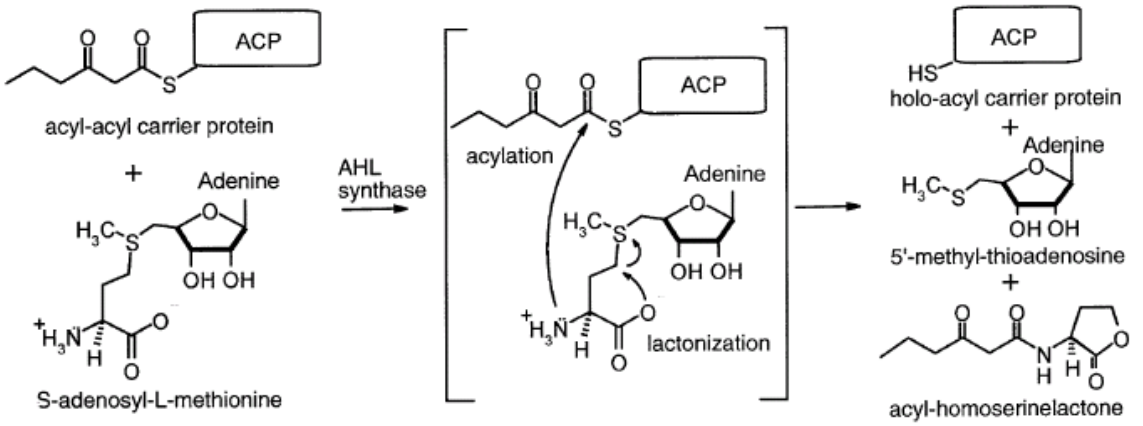


Figure 3. The schematic drawing illustrates the general features of the AHSL synthesis reaction. Two substrates, acyl-ACP and SAM, bind to the AHSL synthase. After the acylation and lactonization reactions, the product AHSL and holo-ACP and 5'-methylthioadenosine are released. Adapted from Watson *et al*. 2002.

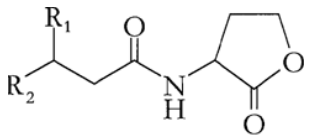
There is considerable structural variety between AHSL signaling molecules from different bacteria and also between AHSLs synthesized by the same bacterium (Table 2). The acyl chain length can vary between four and eighteen carbons and the third carbon in the acyl chain may be a fully oxidized carbonyl, 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.

Bacteria	Regulators	Chain length (R_2)	β R-group (R_1)	Target function	References
Agrobacterium tumefaciens	TraI-TraR	C8 (4)	=O	Conjugal transfer	Zhang *et al*. 1993
Erwinia carotovora subsp. *carotovora*	ExpI-ExpR	C6 (2)	=O	Extracellular enzymes and antibiotics	Pirhonen *et al*. 1993; Jones *et al*. 1993; McGowan *et al*. 1995
		C8 (4)	=O		
Pantoea stewartii	EsaI-EsaR	C6 (2)	=O	Exopolysaccharide	Von Bodman and Farrand, 1995
Pseudomonas aeruginosa	LasI-LasR	C12 (8)	=O	Virulence and biofilm development	Pearson *et al*. 1997
	RhlI-RhlR	C4 (0)	−H	Virulence and rhamnolipids	Pearson *et al*. 1997
Rhizobium leguminosarum	CinI-CinR	C14:1 (10)	−OH	Rhizome interactions, root nodulation	Lithgow *et al*. 2000
Sinorhizobium meliloti	SinI-SinR	C16:1 (12)	−H	Exopolysaccharide	Marketon *et al*. 2002
		C18:1 (14)	−H	Exopolysaccharide	Marketon *et al*. 2002
Vibrio fischeri	LuxI-LuxR	C6 (2)	=O	Bioluminescence	Eberhard *et al*. 1981



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 *esaR* 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 *esaR* mutant produces higher levels of the virulence factor exopolysaccharide compared to the wild-type (von Bodman *et al.* 1998).

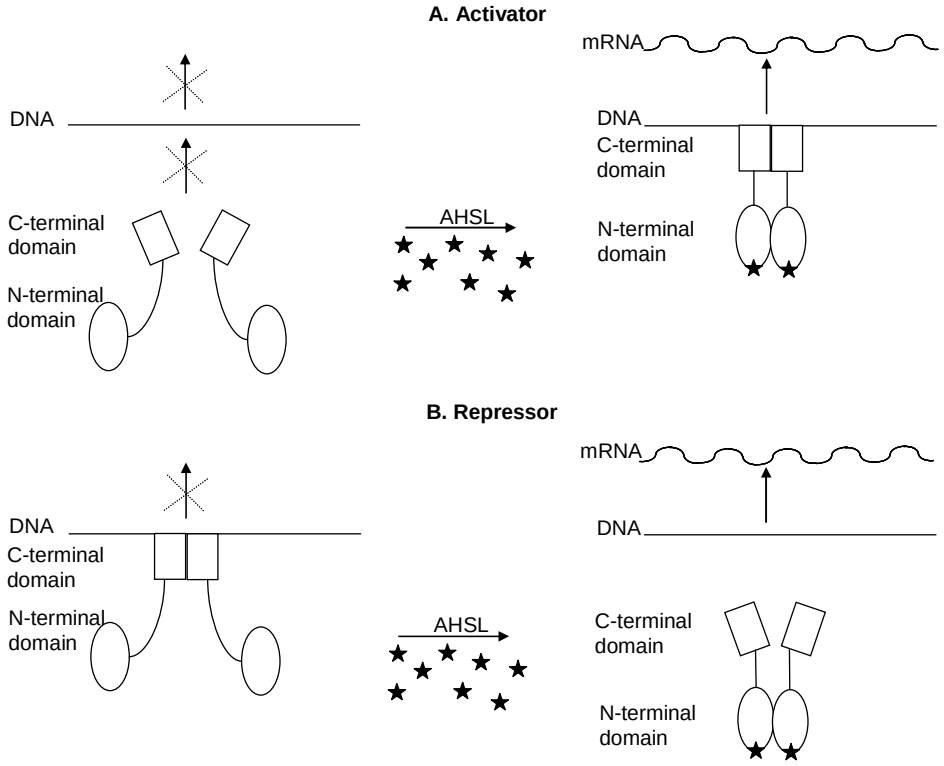


Figure 4. Schematic presentation of the structural modifications induced by AHSL binding on the activators and repressors of the LuxR-type family. **A.** Activators: In the absence of AHSL, the N-terminal domain masks the C-terminal DNA-binding domain and thus interferes with the DNA binding. Binding of the appropriate ligand to the activator induce conformational changes that lead to exposure of the DNA-binding domain and subsequent DNA-binding. **B.** Repressors: 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 *esaR* 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 *Pantoea* 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, ATTn10 and MS1, Ecc71 and *Eca* strain SCRI1043, 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 AhlI (Cui *et al*. 2005) or HslI (Cui *et al*. 1995), in SCC3193 ExpI (Pirhonen *et al*. 1993) and in the *Eca* strain SCRI1043 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 SCRI1043, 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 carbapenem. The *carR* gene is located immediately upstream of the *carA-H* biosynthetic operon, which encodes proteins required for carbapenem biosynthesis and for the carbapenem 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 carbapenem production (McGowan *et al*. 2005). Environmental factors such as temperature, pH and carbon source also affect synthesis of carbapenem. The environmental influence on carbapenem 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 carbapenem 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 carbapenem (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; Mäe *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 impedes 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.

Strain	**Genotype/Relevant characteristics**	**Reference**
E. coli		
S17-1 λ pir	*recA thi pro hsdR⁻ M⁺* RP4:2-Tc:MuKm Tn7 λ pir, Tp^R Sm^R	Miller and Mekalanos, 1988
DH5α	*endAI hsdR17 supE44 thi-1 gyrA96 relA1 ΔlacU169* (*ϕ80dlacΔM15*)	Hanahan, 1983
JM109	e14⁻(McrA-) *recA1 endA1 gyrA96 thi-1 hsdR17*($r_{K-}m_{K+}$) *supE44 relA1* Δ(*lac*-	Yanish-Perron *et al*. 1985
Erwinia carotovora subsp. *carotovora* strains		
SCC1	Wild-type	Pirhonen *et al*. 1988
SCC3193	Wild-type	Saarilahti and Palva, 1986
SCC3065	*expI*::km in SCC3193 background, Km^R	Pirhonen *et al*. 1991
SCC6005	*expIexpR1*::*cat* in SCC3193 background, Cm^R	Andersson *et al*. 2000
expI rsmA mutant	*rsmA* in SCC3065 background, Km^R	Andersson (unpublished)
SCC5003	*expR1*::*cat* in SCC3193 background, Cm^R	Andersson *et al*. 2000
SCC905	*expR2*::*cat* in SCC3193 background, Cm^R	II
SCC905	*expR2*::*km* in SCC3193 background, Km^R	II
SCC906	*expR2*::*km* in SCC6005 background, Km^R Cm^R	II
SCC907	*expR2*::*km* in SCC5003 background, Km^R	II
SCC908	*expR2*::*cat* in SCC3065 background, Cm^R	II
SCC802-1	*ferE*::miniTn5*cat*::*gusA* in SCC3193 background, Cm^R	III
SCC802	*ferE*:: miniTn5*cat*::*gusA* in SCC3065 background, Km^R Cm^R	III
SCC804-1	*hor*:: miniTn5*cat*::*gusA* in SCC3193 background, Cm^R	III
SCC804	*hor*:: miniTn5*cat*::*gusA* in SCC3065 background, Km^R Cm^R	III

Table 4. Plasmids used in this study.

Plasmid	Relevant property	Reference/Source
pSB402	pBR322 with *luxRI'* and a promoterless *luxCDABE* cassette, Amp^R	Guard-Petter,1998
pBluescript SK+	Cloning vector, Amp^R	Stratagene
pGP704	Suicide vector, Amp^R	Miller and Mekalanos, 1988
pPRG	pUT-mini-Tn5Cm^R::*gusA*	Marits *et al*. 1999
pQE30	Expression vector, Amp^R	Qiagen
pGUS102	pBR322 with promoterless *uidA* from *E. coli*, Amp^R	Andersson *et al*. 2000
$pExpI_{SCC3193}$	$exp1_{SCC3193}$ cloned into pBSK+	Pirhonen *et al*. 1993
$pExpI_{SCC1}$ in pQE30	$expI_{SCC1}$ cloned into pQE30 *Bam* HI and *Hind* III	I
$pExpI_{SCC1}$ in pBSK+	$expI_{SCC1}$ cloned into pBSK+ *Bam* HI and *Hind* III	I
$pExpI_{SCC1}$ L67S	$expI_{SCC1}$ L67S in pBSK+	I
$pExpI_{SCC1}$ F69L	$expI_{SCC1}$ F69L in pBSK+	I
$pExpI_{SCC1}$ L123F	$expI_{SCC1}$ L123F in pBSK+	I
$pExpI_{SCC1}$ S126A	$expI_{SCC1}$ S126A in pBSK+	I
$pExpI_{SCC1}$ M127T	$expI_{SCC1}$ M127T in pBSK+	I
$pExpI_{SCC1}$ L67S M127T	$expI_{SCC1}$ L67S M127T in pBSK+	I
$pExpI_{SCC1}$ F69L M127T	$expI_{SCC1}$ F69L M127T in pBSK+	I
$pExpI_{SCC1}$ L67S F69L M127T	$expI_{SCC1}$ L67S F69L M127T in pBSK+	I
$pExpI_{SCC1}$ S126A M127T	$expI_{SCC1}$ S126A M127T in pBSK+	I
$pExpI_{SCC1}$ L67S S126A M127T	$expI_{SCC1}$ L67S S126A M127T in pBSK+	I
$pExpI_{SCC1}$ F69L S126A M127T	$expI_{SCC1}$ F69L S126A M127T in pBSK+	I
$pExpI_{SCC1}$ L67S F69L S126A M127T	$expI_{SCC1}$ L67S F69L S126A M127T in pBSK+	I
$pExpI_{SCC1}$ L67S F69L L123F M127T	$expI_{SCC1}$ L67S F69L L123F M127T in pBSK+	I
$pExpI_{SCC1}$ L67S F69L L123F S126A M127T	$expI_{SCC1}$ L67S F69L L123F S126A M127T in pBSK+	I
$pExpI_{SCC3193}$ T127L	$expI_{SCC3193}$ T127L in pBSK+	I
$pExpI_{SCC3193}$ T127M	$expI_{SCC3193}$ T127M in pBSK+	I
$pExpI_{SCC1}$ M127T pQE30	$expI_{SCC1}$ M127T in pQE30	I
$pExpI_{SCC1}$ F69L M127T pQE30	$expI_{SCC1}$ F69L M127T in pQE30	I
pSMS20	$expR1_{SCC3193}$ cloned into pQE30 *Eco* RI and *Bam* HI	II
pSMS21	$expR2_{SCC3193}$ cloned into pQE30 *Eco* RI and *Bam* HI	II
pSMS22	$expR_{SCC1}$ cloned into pQE30 *Eco* RI and *Bam* HI	II
pSMS18	$rsmA_{SCC3193}$ promoter (189 nt) and partial CDS (67 nt) cloned into pGUS102 *Sal* I and *Hind* III	II
pSMS100	889 bp DNA fragment containing upstream region of *expR2*	II
pSMS103	*cat* gene and *km* gene cloned into pSMS100	II
pSMS104	$expR2_{SCC3193}$::*km*, $expR2_{SCC3193}$::*cat* cloned into pBluescript *Apa* I and *Spe* I	II
pSMS105	$expR2_{SCC3193}$::*km*, $expR2_{SCC3193}$::*cat* cloned into pGP704 *Apa* I and *Spe* I	II
pSMS30	*ferE* cloned into pQE30 *Eco* RI and *Bam* HI	III
pSMS31	*hor* cloned into pQE30 *Eco* RI and *Bam* HI	III

Table 5. Methods used in this study.

Method	Described and used in
AHSL extraction	I
Arbitrary PCR	II
Assay for AHSL binding	II
Assay for oxidative stress tolerance	III
Bacterial survival test *in planta*	III
Bioluminescence assay	II
DNA sequencing	I, II, III
Extracellular enzyme assays	I, II, III
β-Glucuronidase (GUS) assay	II, III
Homology modelling	I
Infection of *Arabidopsis* with *Ecc*	II, III
Infection of potato with *Ecc*	I, II, III
Liquid chromatography-mass spectrometry	I
Marker exchange mutagenesis	II
Molecular cloning techniques	I, II, III
Northern blot analysis	II, III
Phage library screening	II
Plant growth conditions	II, III
Protein expression in *E. coli*	I
RNA isolation	II, III
Site-directed mutagenesis	I
T4GT7 transduction	III
Transposon mutagenesis	III
Virulence test	I, II, III
Western blot analysis	I

D. RESULTS AND DISCUSSION

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 then 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 $expI_{SCC3193}$ and $expI_{SCC1}$ 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 then 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 AHSL 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 *Ecc* strain produces 3-oxo-C6-HSL and the other one 3-oxo-C8-HSL. We aligned closely related ExpI proteins, producing AHSLs with different chain lengths augmented with the crystal structure of the homologous EsaI of *P. stewartii* (Watson *et al*. 2002). The 3-oxo-C6-HSL producing EsaI of *P. stewartii*, CarI of *Ecc* GS101, ExpI of *Ecc* of SCC1 and ExpI of *Echr* 3937, and the 3-oxo-C8-HSL producing ExpI of *Ecc* SCC3193 and ExpI of *Ec* CFBP6272, were compared. A clear pattern of amino acid residues differentiating the two types of ExpI proteins, which produce either 3-oxo-C6-HSL or 3-oxo-C8-HSL was identified in the close proximity of the putative acyl substrate binding pocket determined in the crystal structure of EsaI (Watson *et al*. 2002) (I, Figure 3). In the $ExpI_{SCC1}$ protein producing 3-oxo-C6-HSL, the identified residues were Leu-67, Phe-69, Leu-123 and Met-127, while the corresponding residues in the $ExpI_{SCC3193}$ protein producing 3-oxo-C8-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 $ExpI_{SCC1}$ to the corresponding residue in the $ExpI_{SCC3193}$. 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 *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-C6-HSL producing $ExpI_{SCC1}$ to a 3-oxo-C8-HSL producing ExpI, however the amount of AHSL was drastically reduced. To confirm the importantance of residue 127, an opposite change of T127M in $ExpI_{SCC3193}$ was generated. This led to a similar effect with the former 3-oxo-C8-HSL producing $ExpI_{SCC3193}$, subsequently producing 3-oxo-C6-HSL in a similarly reduced amount. The single mutations L67S and F69L in $ExpI_{SCC1}$ did not affect the type of AHSL. However, these mutations reduced clearly the amount of AHSL produced. The single mutations L123F and S126A in $ExpI_{SCC1}$ did not alter the amount nor the type of AHSLs produced.

1.3. Mutations causing changes in bacterial communication

The M127T change in $ExpI_{SCC1}$ changed the communication language, but the AHSL amount generated (10 nM) was too low to restore the cellulase activity in the *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 $ExpI_{SCC1}$ 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 $ExpI_{SCC3193}$.

The obtained results are in accordance with the EsaI crystal structure, showing that in $ExpI_{SCC1}$ 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 *et al.* 2002; Gould *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_6 -tagged variants of $ExpI_{SCC1}$ 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 Salmond, 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 SCRI1043 and ExpR2 of *Ecc* strain Ecc71 (Burr *et al*. 2005; Cui *et al*. 2006). Contrary to our findings, mutations in $virR_{Eca}$ and $expR2_{Ecc71}$ 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 *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 *expI expR2* mutant bound only to the cognate AHSL 3-oxo-C8-HSL, while the *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 *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 *Ecc* the expression of *rsmA*, the global negative regulator of virulence, was affected by the presence of AHSL. The level of *rsmA* mRNA was highly increased in the *expI* mutant compared to the wild-type (Cui *et al.* 1995; Mäe *et al.* 2001).This prompted us to investigate in the role of ExpR1 and ExpR2 in the expression of *rsmA.*

For this purpose, we tested the activity of an *rsmA-gusA* promoter fusion in different mutant backgrounds (II, Figure 5). The *expI* mutant, grown either without cognate AHSL or with the addition of the non-cognate 3-oxo-C6-HSL, showed increased activity of the *rsmA-gusA* promoter fusion, while this was suppressed with 3-oxo-C8-HSL. The *expI expR2* double mutant acted in the same manner. We suggest this is due to the presence of the ExpR1 protein, which activates the *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 *rsmA* expression. On the other hand, in the *expI expR1* mutant, the *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 *rsmA-gusA* promoter fusion activation. In the *expI expR1 expR2* triple mutant, the activation of *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 *rsmA.* The addition of AHSLs suppressed the activation of *rsmA* transcription. This suppression acted in accordance with the ligand binding specificity identified for the ExpR1 and ExpR2 proteins. Furthermore, the *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 $ExpR_{71}$ binds to the promoter of *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 *expI 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 $expR1_{SCC1}$ to the *expI 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 $expR1_{SCC1}$ to the *expI* mutant and the *expI 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 phenylacetate-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 synthesising 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 e*xpI 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 H_2O_2 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 H_2O_2 on the *expI* mutant. These results demonstrated that the *expI* mutant was clearly sensitive to H_2O_2 with 40 mM H_2O_2 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_2O_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 *expI*, *expI expR1*, *expI expR2* and *expI 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.

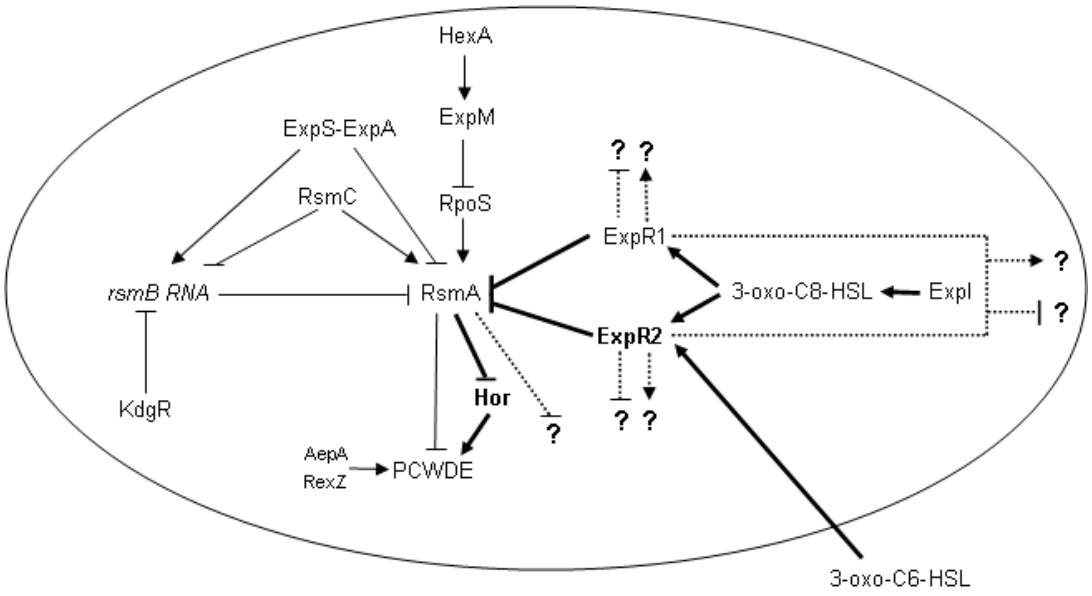


Figure 5. Schematic representation of the complex regulatory network involved in controlling PCWDE synthesis in *E. carotovora* subsp. *carotovora* SCC3193. Arrow heads indicate positive regulation and flattened ends denote negative regulation. Adapted and modified from Whitehead *et al*. 2002 according to recent findings of this thesis study. Bolded lines indicate new findings from this study and dashed lines indicate possible future studies.

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?

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