Regulatory networks controlling virulence in the plant pathogen *Erwinia carotovora* ssp. *carotovora*

Heidi Hyytiäinen

Department of Biological and Environmental Sciences
Faculty of Biosciences
University of Helsinki

Academic dissertation
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Supervisor

Professor E. Tapio Palva
Department of Biological and Environmental Sciences
University of Helsinki, Finland

Reviewers

Docent Elina Roine
Department of Biological and Environmental Sciences
Institute of Biotechnology
University of Helsinki, Finland

Professor Mikael Skurnik
Department of Bacteriology and Immunology
Faculty of Medicine
University of Helsinki, Finland

Opponent

Professor Per Saris
Department of Applied Chemistry and Microbiology
Faculty of Agriculture and Forestry
University of Helsinki, Finland

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LIST OF ORIGINAL ARTICLES

This thesis is based on the following original articles referred to in the text by their Roman numerals. Some unpublished data are also included.

I. **Hyytiäinen,** H., Montesano, M. and Palva, E.T. (2001) Global regulators ExpA (GacA) and KdgR modulate extracellular enzyme gene expression through the RsmA-*rsmB* system in *Erwinia carotovora* subsp. *carotovora*. *Mol Plant-Microbe Interact* **14:** 931-938.

II. **Hyytiäinen**, H., Sjöblom, S., Palomäki, T., Tuikkala, A. and Palva, E.T. (2003) The PmrA-PmrB two-component system responding to acidic pH and iron controls virulence in the plant pathogen *Erwinia carotovora* ssp. *carotovora*. *Mol Microbiol* **50:** 795-807.

III. **Hyytiäinen**, H., Saarilahti, H. and Palva, E.T. (2005) The role of a small RyhB-like RNA in the plant pathogen *Erwinia carotovora* ssp. *carotovora* (submitted).

ABBREVIATIONS

aa	amino acid
AHL	*N*-acylhomoserine lactone
bp	base pairs
cAMP	cyclic AMP
Cel	cellulase
CRP	cyclic AMP receptor protein
Eca	*Erwinia carotovora* ssp. *atroseptica*
Ecc	*Erwinia carotovora* ssp. *carotovora*
Echr	*Erwinia chrysanthemi*
HK	histidine kinase
HR	hypersensitive response
HTH	helix-turn-helix
kDa	kilodalton
LPS	lipopolysaccharide
Nep	necrosis- and ethylene-inducing peptide
ORF	open reading frame
PCR	polymerase chain reaction
PCWDE	plant cell wall–degrading enzymes
Peh	polygalacturonase
Pel	pectate lyase
PGA	polygalacturonic acid
PM	polymyxin B
Pme	pectin methylesterase
Pnl	pectin lyase
Prt	protease
RFLP	restriction fragment length polymorphism
RR	response regulator
SPI	*Salmonella* pathogenicity island
TCS	two-component regulatory system
UTR	untranslated region

ABBREVIATIONS FOR GENE MNEMONIC

aep	**a**ctivation of **e**xtracellular protein **p**roduction
air	**a**ttachment and **i**ron **r**egulation
bar	**b**acterial **a**daptive **r**esponses
car	**car**bapenem antibiotic
cel	**cel**lulase
csr	**c**arbon **s**torage **r**egulator
ctv	**c**aro**t**o**v**oricin synthesis
cyt	**cyt**idine catabolism
dsb	**d**i**s**ulfide **b**ond formation
exp	**ex**tracellular enzyme **p**roduction
fur	**f**erric **u**ptake **r**egulator
gac	**g**lobal **a**ctivator of **c**yanide and antibiotic production
grr	**g**lobal **r**esponse **r**egulation
hex	**h**yperproduction of **ex**oenzymes
hil	**h**yper**i**nvasion **l**ocus
hor	**ho**mologue of ***rap***
hrp	**h**ypersensitive **r**eaction and **p**athogenicity
hsl	**h**omo**s**erine **l**actone
lss	cellular **l**y**s**i**s**
peh	**p**olygalacturonase
pel	**pe**ctate **l**yase
pmr	**p**oly**m**yxin **r**esistance
pnl	**p**ecti**n** **l**yase
prt	**pr**o**t**ease
rap	**r**egulation of **a**ntibiotic and **p**igment
rdg	**r**egulator of **d**amage-inducible **g**enes
rex	**r**egulator of **ex**oenzymes
rpf	**r**egulator of **p**athogenicity **f**actors
rpo	**R**NA **po**lymerase
rsm	**r**egulator of **s**econdary **m**etabolism
sir	**S**almonella **i**nvasion **r**egulator
sod	**s**uper**o**xide **d**ismutase
uvr	**UV r**esistance
var	**v**irulence **a**ssociated **r**egulator

ABSTRACT

Erwinia carotovora ssp. *carotovora* (*Ecc*) is a Gram-negative enterobacterium that causes soft rot in potato and other plants. The main virulence factors, secreted extracellular enzymes, degrade the plant cell wall, leading to plant tissue maceration. Extracellular enzymes, cellulases, proteases and pectinolytic enzymes are subject to complex regulatory networks including quorum sensing, two-component systems, sigma factors and regulatory RNAs. One of the most important regulatory systems activating the production of extracellular enzymes and virulence in *Ecc* is the two-component system ExpS-ExpA. ExpS is a cytoplasmic membrane-associated protein suggested to activate its cognate partner ExpA, a response regulator protein. However, the signal activating this two-component system is unknown, and the target genes of the DNA-binding protein ExpA remain to be unravelled. ExpA and its orthologues are currently thought to mediate their effects through other regulatory components in diverse bacterial species.

The aim here was to characterize regulatory components acting in concert with ExpA or serving as downstream regulators of ExpA in the control of extracellular enzyme genes. We applied a second-site transposon mutagenesis to an avirulent *expA* mutant to identify genes whose inactivation results in suppression of an extracellular enzyme-negative phenotype of the *expA* mutant. We identified an insertion in the *kdgR* gene, which was earlier characterized to repress expression of extracellular enzyme genes and a small regulatory RsmB RNA. Our results indicated that both regulatory proteins ExpA and KdgR affect expression of the RsmA-RsmB RNA regulatory system and that activation of extracellular enzyme genes by ExpA is partially mediated through this system. However, other mechanisms are likely also involved in activation of virulence factors by ExpA.

In a second study, we identified a novel two-component system for plant pathogenic bacteria, PmrA-PmrB, which affects production of extracellular enzymes, virulence and bacterial survival *in planta*. The PmrA-PmrB regulatory system was suggested to modify bacterial lipopolysaccharide, thus promoting bacterial resistance to plant-derived antimicrobial peptides. Furthermore, a functional PmrA was necessary for survival excess of iron in conditions where pH was mildly acidic. However, the putative interaction between the two-component systems ExpS-ExpA and PmrA-PmrB requires further clarification. We also identified a novel small functional RNA RyhB, which affects expression of the *celV1* gene encoding a cellulase. Expression of RyhB RNA was induced in iron-limiting conditions, suggesting its role in iron homeostasis. However, *Ecc* RyhB RNA was not involved in regulation of the *sdhCDAB* operon encoding succinate dehydrogenase enzyme, which requires Fe^{2+} ions for activity. Strikingly, ExpA was found to regulate the *sdhCDAB* operon, and inactivation of *expA* resulted in inability to utilize C_4-dicarboxylates succinate or fumarate, which act as chemoattractants for some plant-associated bacteria.

In conclusion, our findings indicate that response regulator ExpA mediates its effects through another system, RsmA-RsmB RNA, and that the complex regulatory network includes novel mechanisms controlling virulence such as PmrA-PmrB and RyhB RNA. Furthermore, although ExpA is a central activator for expression of extracellular enzyme genes, it also has a role in another regulatory pathway, carbon metabolism.

A. INTRODUCTION

1. The genus *Erwinia*

The members of *Erwinia* sp. belong to the *Enterobacteriaceae* family and form a heterologous group of Gram-negative plant pathogens. Three economically important groups are *Erwinia carotovora* ssp. carotovora (*Ecc*), *Erwinia carotovora* ssp. *atroseptica* (*Eca*) and *Erwinia chrysanthemi* (*Echr*), which cause soft rot in potato as well as in other crops (Perombelon, 2002; Toth, 2003). The soft rot erwinias are necrotrophic intercellular pathogens capable of killing plant tissues rapidly, although their aggressiveness varies (Salmond *et al*., 1994; Alfano and Collmer, 1996). They have been proposed to be reclassified as the genus *Pectobacterium* based on their 16S rDNA, but this nomenclature has not been widely accepted (Perombelon, 2002; Toth, 2003).

These facultative anaerobic bacteria share many similar characteristics, including their wide host range and disease symptoms, and most importantly, their capability to produce a large arsenal of different plant cell wall-degrading enzymes that play a central role in pathogenesis. The *Eca* strains are differentiated from other *Erwinia carotovora* strains by their inability to grow at temperatures above 36ºC and by their production of acid from α-methyl glucoside and reducing substances from sucrose (De Boer, 2003). The subspecies can be differentiated by analysing lipopolysaccharide O-antigen, phage sensitivity or PCR-RFLP profiles. However, significant phenotypic differences are present among strains within subspecies (Perombelon, 2002; De Boer, 2003). When the genome sequences of *Eca* and *Echr* are both available, allowing comparison between each other and with known genes of *Erwinia* type strains, the genetic differences related to extracellular enzymes, type III effectors and other pathogenicity factors reveal themselves (Bell *et al*., 2002; 2004; Toth *et al*., 2003). This introduction will focus mostly on research performed at the level of subspecies *carotovora*.

1.1. *Ecc* and pathogenicity

Ecc is an opportunistic pathogen whose virulence is dependent on its interactions with the host and the environment. Under favourable conditions, this plant pathogen expresses virulence factors such as extracellular enzymes. *Ecc* strains of soft-rot erwinias have the broadest host range and the ability to infect plants in both subtropical and temperate regions, and these features are important in their survival (Toth, 2003). On the other hand, their inability to compete in rotting plant tissues with *Eca* or *Echr* or even with saprophytic bacteria has sometimes been considered their weakness (Perombelon, 2002). The environmental factors affecting development of soft-rot symptoms are freely available water, temperature, oxygen tension and available nutrients. Thus, an increase in free water may lead to a decreased amount of oxygen, favouring the pathogenicity of *Ecc* and impairing oxygen-dependent resistance systems of plants (Perombelon, 2002; Toth, 2003).

At the beginning of the 1980s, a set of Finnish *Ecc* and *Eca* isolates was obtained from infected potatoes, and the serogroups were determined in conjunction with a test of occurrence bacteriophage T4 receptor (Pirhonen and Palva, 1988). The *Ecc* strain SCC3193 was unique among the other *Ecc* strains in that it was sensitive to T4 infection, thus allowing

transduction of chromosomal genes and plasmids. Based on this and obvious pathogenicity characteristics, i.e. extracellular enzymes and cell density-dependent quorum sensing system, strain SCC3193 was selected for further studies (Pirhonen *et al*., 1988; 1991; 1993). Studies have subsequently shown that while the Finnish *Ecc* isolates share many common features, certain critical differences are present in their pathogenicity-related characters (Rantakari *et al*., 2001; Mattinen *et al*., 2004).

1.2. Extracellular enzymes

It is essential that virulence factors are efficiently delivered from bacterial cells to the external milieu. For this purpose, *Ecc* employs specific secretion systems. Plant cell wall-degrading enzymes, pectinolytic enzymes and cellulases are secreted by the type II system, and proteases by the type I system (Toth *et al*., 2003).

1.2.1. Pectinolytic enzymes

Pectinolytic enzymes, also known as pectinases, are the main extracellular enzymes involved in disease development. Pectinases, including pectate lyases (Pel), pectin lyases (Pnl), polygalacturonases (Peh) and pectin methylesterases (Pme), break down and utilize pectins, causing tissue collapse, cell damage and cell leakage (Toth *et al*., 2003). Many of them exist in multiple forms, isoenzymes, encoded by independent genes and are considered to evolve from sequential rounds of gene duplication (Toth *et al*., 2003). The characterized genes encoding pectinases from different *Ecc* strains are shown in Table 1.

Table 1. Pectinolytic extracellular enzyme genes characterized from *Ecc* strains.

Strain	Gene	Reference
ER	*pel1*	Ito *et al*. (1988)
	pelX	Ito *et al*. (1988)
	pel2	Yoshida *et al*. (1992)
	pel3	Yoshida *et al*., (1991)
	pnl	Oshini *et al*. (1991)
ECC71	*pel-1*	Chatterjee *et al*. (1995a)
	pel-3	Liu *et al*. (1994a)
	pnlA	McEvoy *et al*. (1990); Chatterjee *et al*. (1991)
	peh-1	Liu *et al*. (1994a)
SCRI193	*pelB*	Hinton *et al*. (1989)
	pelC	Hinton *et al*. (1989)
	peh	Hinton *et al*. (1990)
SCC3193	*pelB*	Heikinheimo *et al*. (1995)
	pehA	Saarilahti *et al*. (1990b)
EC153	*pel153*	Trollinger *et al*. (1989)

1.2.1.1 Pectate and pectin lyases

Pectate lyases (Pel; EC 4.2.2.2) cleave α-1,4-glycosidic linkages in pectate by ß-eliminating glycosidic bonds and producing unsaturated products (Barras *et al*., 1994). However, the number of *pel* genes varies between species, subspecies and strains (Toth *et al*., 2003). Four major Pels (PelA, B, C, D) and few minor Pels Have been characterized from *Ecc* strains (Ito *et al*., 1988; Hinton *et al*., 1989; Yoshida *et al*., 1991; 1992; Liu *et al*., 1994a; Chatterjee *et al*., 1995a; Heikinheimo *et al*., 1995). Most Pels function inefficiently at acidic pH, reflecting the conditions encountered by invading bacteria *in planta* (Py *et al*., 1998). Therefore, while PelB (Pel2) and PelC (Pel3) of *Ecc* are considered as the main pectate lyases responsible for plant tissue maceration, the combined action of other extracellular enzymes is important in the plant apoplast, where the pH is around 6 (Perombelon, 2002).

A novel type of pectate lyase was characterized from $Ecc_{SCC3193}$ by isoelectric focusing (Heikinheimo *et al.*, 1995). The *pelB* gene encoding a 35-kDa processed peptide showed 93% amino acid identity to the Pel-3_{Ecc71} (PelC) but, unlike other Pels, also had distinct substrate specificity and did not require Ca^{2+} ions for activity (Liu *et al*., 1994a; Heikinheimo *et al*., 1995). In addition, the Pel isoenzyme profile achieved by isoelectric focusing revealed that the most dominant Pels in $Ecc_{SCC3193}$ are PelC and PelD.

Pectin lyases (Pnl; EC 4.2.2.10), known also as pectin methyltranseliminases, favour and cleave highly methoxylated pectin. Pnl activity is suggested to increase following exposure to plant- derived DNA-damaging agents (McEvoy *et al*., 1990). The *pnlA* gene, which encodes a 32-kDa protein without any signal sequence, was isolated from Ecc_{Ecc71} (McEvoy *et al*., 1990; Chatterjee *et al*., 1991). Transcriptional activation of the *pnlA* required the functional RecA protein, and the Pnl protein was suggested to leak semispecifically through the lipid bilayers (Chatterjee *et al*., 1991). The *pnlA* gene was shown to also exist in other *Ecc* but not in *Echr* strains (McEvoy *et al*., 1990).

1.2.1.2 Polygalacturonases

Polygalacturonases (Peh; EC 3.2.1.15) attack the α-1,4-glycosidic linkages in pectate, cleaving them by hydrolysis producing saturated products (Barras *et al*., 1994). Two different polygalacturonases have been characterized, the exo- and endo-Peh; the former releases digalacturonic acid from the free ends of polygalacturonic acid (PGA), and the latter cleaves the internal linkages and is only found in *Ecc* strains. The published endo-Pehs from *Ecc* strains show high homology with each other, 95% identity at the amino-acid level (Hinton *et al*., 1990; Saarilahti *et al*., 1990b; Liu *et al*., 1994a). The $pehA_{SCC3193}$ gene encodes an approximately 40-kDa polypeptide, with an optimal pH of 5.5 and an optimal temperature of 35-45ºC (Saarilahti *et al*., 1990b). The production of $Peh_{SCC3193}$ was suggested to be important at the initial phase of infection, and its production was clearly repressed by calcium, which reflects breakage of plant cell walls (Saarilahti *et al*., 1992; Flego *et al*., 1997). Conversely, the Peh characterized from $Ecc_{SCRI193}$ was non-inducible by PGA and suggested to be expressed constitutively (Hinton *et al*., 1990).

1.2.2. Cellulases

Cellulases (Cel; EC 3.2.1.4) act on plant cell walls by endohydrolysis of 1,4-β-D-glucosidic linkages in cellulose, lichenin and cereal β-D-glucans. Their optimal pH is near 7, and cellulases are supposed to act in synergy with other extracellular enzymes to attack the primary and secondary cell walls of plants (Barras *et al*., 1994; Toth *et al*., 2003).

The *celS* gene encoding a 264-aa polypeptide was isolated from a genomic library of $Ecc_{SCC3193}$ (Saarilahti *et al.*, 1990a). The CelS contained a 32-aa cleavable signal peptide, and the mature CelS had a molecular mass of 27 kDa. Comparison with other cellulases showed low sequence similarities to the known cellulases, and thus, $CelS_{SCC3193}$ was classified as the first member of a novel cellulase family (Saarilahti *et al.*, 1990a). Another cellulase, described as a major cellulase of *Ecc*, was identified from $Ecc_{SCRI193}$ and $Ecc_{SCC3193}$ (Cooper and Salmond, 1993; Walker *et al*., 1994; Mäe *et al*., 1995). The $celV_{SCRI193}$ gene encoding a 55-kDa polypeptide was identified by expressing the genomic library of *Ecc* in *Escherichia coli* and screening cellulase-positive clones (Cooper and Salmond, 1993). The homology comparison between the N-terminal catalytic domain and the C-terminal cellulose-binding domain of the CelV with other cellulases suggested that it had evolved from several different cellulase families (Cooper and Salmond, 1993). Inactivation of the $celV_{SCRI193}$ gene significantly reduced the maceration capacity of potato tissue as compared with the wild type (Walker *et al*., 1994).

The $celV1_{SCC3193}$ gene encoded a cellulase with 93% amino acid identity to $CelV_{SCRI193}$ (Mäe *et al*., 1995). The $celV1_{SCC3193}$ mutant showed reduced maceration capacity on tobacco leaves compared with the wild type, especially at the early stages of infection. Inactivation of the *celS* gene in the *celV1* mutant background did not lead to any further decrease in virulence, suggesting that $CelS_{SCC3193}$ might be important in infection of different host plants or during saprophytic growth (Mäe *et al*., 1995).

The CelA and CelB proteins able to hydrolyse carboxymethylcellulose were characterized from Ecc_{LY34} (Park *et al*., 1997). CelA showed 96% and 92% amino acid identity to $CelV_{SCRI193}$ and $CelV1_{SCC3193}$, respectively. CelB was 84% identical to the $CelS_{SCC3193}$ protein, and the results confirmed previous observations that CelB homologues have lower cellulase activity than CelA homologues in *Ecc* (Park *et al*., 1997).

1.2.3. Proteases

Most of the research on *Erwinia* proteases has been accomplished in type I secretion apparatus and regulatory components affecting production of proteases (Py *et al*., 1998). However, inactivation of the *prt1* gene reduced protease activity on gelatine plates by approximately 60% to 80% in the Ecc_{EC14} strain (Kyöstiö *et al*., 1991). The *prt1* gene encoded a 38-kDa protein with similarity to bacterial metalloproteases, but surprisingly, the conserved regions were more homologous to thermolysin of *Bacillus thermoprotelyticus* (58% to 71%) than to protease B of *Echr* (18% to 32%). The authors suggested that differences in secreted proteases might vary in erwinias similarly to differences in pectolytic enzymes (Kyöstiö *et al*., 1991). An extracellular metalloprotease was isolated from the culture medium of *Ecc* (Heilbronn *et al*., 1995). The protease had a molecular mass of 53 kDa and was shown to be different from the Prt1 of Ecc_{EC14} on the basis of the sequenced N-terminus. In addition, it

was able to degrade potato lectin *in vitro*, and the authors suggested that this protease might have a role in degrading plant proteins associated with resistance (Heilbronn *et al*., 1995).

The strain $Ecc_{SCC3193}$ was transposon-mutagenized, and plant extract-inducible genes were isolated (Marits *et al*., 1999). One of the mutants produced undetectable levels of protease and was further characterized to contain an insertion in the *prtW* gene encoding a 51-kDa polypeptide. The sequence analysis of the *prtW* showed over 60% identity to published proteases PrtB, PrtC, PrtA and PrtG of *Echr*, but the previously described proteases of *Ecc* were clearly dissimilar (Marits *et al*., 1999). The $PrtW_{SCC3193}$ contained similar Ca^{2+}- and Zn^{2+}-binding domains as described for *Echr* metalloproteases (Marits *et al*., 1999). The *prtW* mutant was less virulent than the wild-type strain, it retained about 60% of its maceration capacity on potato tubers and 63% of tobacco seedlings showed normal soft-rot symptoms (Marits *et al*., 1999). Expression of the *prtW* gene during the early growth phase was suggested to be necessary for suppressing the plant defense response (Marits *et al*., 1999).

1.3. Other factors affecting pathogenicity

Plant cell wall-degrading enzymes are the main characteristics responsible for soft-rot symptom development. However, other factors also affect the early stage, establishment and progression of infection, as well as responses to host resistance mechanisms (Norman-Setterblad *et al*., 2000). These factors include motility, lipopolysaccharides, siderophores, *hrp* genes, Nep1-like proteins, and factors against oxygen damage or antimicrobial peptides (Hancock *et al*., 1995; García-Olmedo *et al*., 1998; Toth, 2003).

1.3.1. Motility

Motility has been described as a pathogenicity determinant in *Erwinia*, as it appears to be necessary for the successful invasion and infection of potato plants (Perombelon, 2002). However, it is likely less important when infecting potato tuber tissue. While most used *Ecc* type strains have been reported to be non-motile, wild-type $Ecc_{SCC3193}$ is motile (Pirhonen *et al.* 1991; Mukherjee *et al*., 1996). Characterized Mot^- strains have shown reduced virulence in tobacco but produce extracellular enzymes like the wild-type strain (Pirhonen *et al*., 1991).

1.3.2. Lipopolysaccharides

Lipopolysaccharides (LPSs) have apparently diverse roles in bacterial pathogenesis of plants. LPS is an outer membrane component composed of lipid A, core oligosaccharide and O-antigen which may contribute to resistance of bacteria to plant-derived antimicrobial compounds, thus promoting the bacterial infection process. However, LPS recognized by plants can trigger plant defense responses (Dow *et al*., 2000; Newman *et al*., 2002). Gram-negative bacteria have evolved mechanisms to modify the structure of lipid A in different environments, and these modifications can promote resistance to host cationic antimicrobial peptides (Miller *et al*., 2005). In soft-rot erwinias, LPS has been suggested to provide some protection against antimicrobial substances in potato tubers but in general is not considered to play an important specific role in pathogenesis (Perombelon, 2002). Most LPS-defective

mutants of *Ecc*$_{SCC3193}$ remain virulent and a few exhibit a galactose–sensitive phenotype and are avirulent in tobacco (Pirhonen *et al*., 1988; Pirhonen *et al*., 1991; Toth *et al*., 1999).

1.3.3 Siderophores

Iron is distinct from other nutrients, such as carbon, nitrogen, phosphate and sulphate, or other divalent cations because it is not freely available. Therefore, a key role for pathogenic bacteria is iron acquisition, as well as iron homeostasis during pathogenesis (Expert, 1996; 1999; Escolar *et al*., 1999; Ratledge and Dover, 2000; Hantke, 2001). *Erwinia* sp. acquire iron in the host plant by producing iron-chelating factors known as siderophores. In *Erwinia*, siderophores are also postulated have a protective role against oxidative stress, especially against toxic levels of iron that might lead to generation of active oxygen species (Expert, 1999).

Ecc strain W3C105 was reported to produce two different siderophores, chrysobactin and aerobactin (Ishimaru and Loper, 1992; Bull *et al*., 1994), but mutants deficient in the production of either or both siderophores did not differ from the wild-type strain in the capacity to macerate potato tuber tissue or to cause aerial rot in potato (Bull *et al.*, 1996). The iron acquisition systems have not been studied in other *Ecc* strains.

1.3.4. *hrp* genes

Hypersensitive response (HR) is a defense-associated response in plants elicited by microbes, and in Gram-negative bacteria, this ability resides in the *hrp* gene cluster (Alfano and Collmer, 1996). In plant pathogens, the *hrp* genes encoding a type III secretion system have been considered essential for virulence (Hacker and Kaper, 2000; De Boer, 2003). Several *Ecc* strains have been shown to contain a *hrpN* gene encoding a type III effector and the *hrp* cluster encoding several putative *hrp*-specific regulators (Mukherjee *et al*., 1997; Rantakari *et al*., 2001; Chatterjee *et al*., 2002b; 2002c; Lehtimäki *et al*., 2003). The *hrpN*$_{Ecc71}$ mutants retain the ability to macerate celery petioles similar to the wild-type strain (Mukherjee *et al*., 1997), and inactivation of the *hrpC*$_{SCC1}$ encoding an outer membrane pore-forming protein delays symptom development *in planta* (Rantakari *et al*., 2001).

1.3.5. Nep1-like proteins

A new family of proteins, Nep1-like proteins (NLPs), has been identified in several fungi and bacteria (Pemberton and Salmond, 2004). Members of this family share a high degree of sequence similarity and contain proteins that induce a hypersensitive-like death response in a variety of plants. Recently, a similar kind of necrosis-inducing protein, Nip, was characterized from *Ecc*$_{SCC3193}$, which is deficient in the *hrpN* gene product (Mattinen *et al*., 2004). Nip$_{SCC3193}$ was suggested to be functionally similar to HrpN but is unlikely to be secreted through the type III secretion system (Mattinen *et al*., 2004).

2. Two-component signal transduction system

All living cells must sense changes in their environment and respond accordingly (Appleby *et al.*, 1996). Bacteria employ a sophisticated signal transduction strategy known as the two-component regulatory system (TCS). TCSs handle a multitude of signalling tasks, including host detection and invasion leading to symbiosis or pathogenesis; metabolic adaptation to changes in carbon, nitrogen, electron acceptor and phosphate sources; physiological responses to changes in medium osmolarity; chemotaxis; and stress–induced differentiation such as sporulation (Parkinson and Kofoid, 1992).

A numerous set of different TCSs in bacterial species is evident, e.g. *Escherichia coli* K-12 genome encodes 23 classical histidine kinase (HK) proteins, one unorthodox HK protein and five HK proteins containing both the HK and response regulator (RR) domains. In addition, *E. coli* has 32 RR proteins and one histidine–containing phosphotransfer module (HPt) protein (Mizuno, 1997; Oshima *et al*., 2002). Although these systems are presumed to form an intricate signal network, the detailed mechanism of how they interact with each other is largely unknown (Egushi and Utsumi, 2005). Recently, multiple signal transduction cascades have been suggested for *E. coli*, and a new term, bacterial signal transduction network, has emerged (Egushi and Utsumi, 2005; see Figure 1).

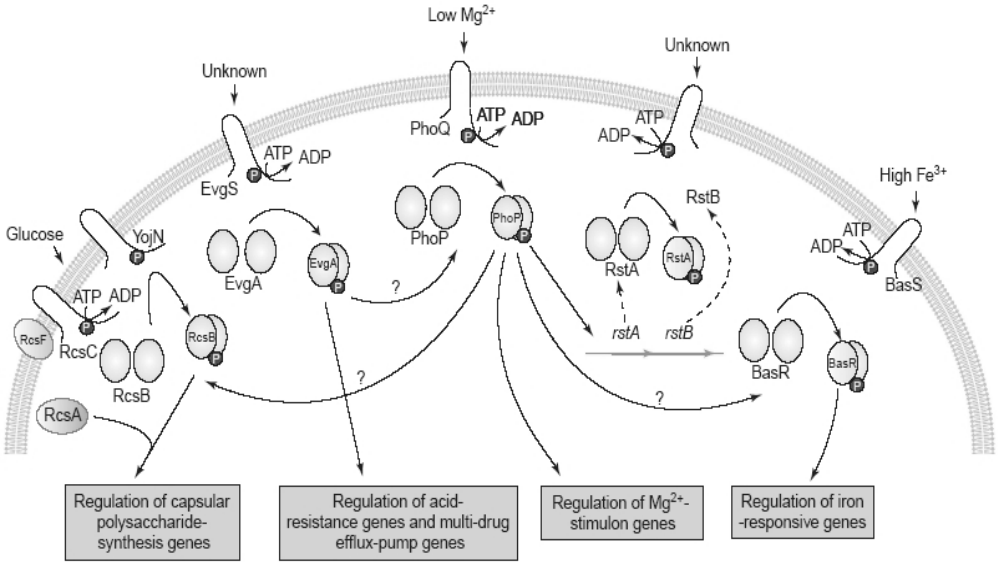


Figure 1. Signal transduction cascade between different two-component systems in *Escherichia coli* (adapted from Egushi and Utsumi, 2005). Question marks indicate that the regulation mechanism of the regulatory cascade is still not clarified.

2.1. Classical two-component system

A classical TCS contains two separate protein components: a sensor HK, which is often located in the cytoplasmic membrane and monitors some environmental parameter, and a cytoplasmic RR, which mediates changes at the cellular level, usually changes in gene expression (Stock *et al*., 1989; Parkinson and Kofoid, 1992; Parkinson, 1993; Hoch and Silhavy, 1995; Hoch, 2000). The sensor HK, the transmitter, regulates the phosphorylation

state of its response-regulating partner in two ways (Parkinson, 1993). First, HK has autokinase activity and, after autophosphorylation at a histidine residue by ATP, the product phosphohistidine serves as an intermediate for transfer of the phosphoryl group to an aspartate residue in the RR, the receiver. Second, some HK proteins have additional phosphatase activity towards their partner RR, thus controlling the level of RR phosphorylation.

2.2. Unorthodox histidine kinase

A more complex TCS has a so-called unorthodox type of HK, which contains additional histidine and aspartate residues, sometimes an additional receiver and sometimes HPt domains. Such modular architecture in HK may allow transduction, e.g. two types of stimuli via different phosphorylation pathways (His_{SK}-Asp_{SK}-His_{SK}-Asp_{RR}), and even cross-talk with their non-cognate RR partners (Perraud *et al*., 1999). However, the presence of several phosphorylation steps in one protein has also been suggested to increase signalling efficiency and reduce non-specific cross-talk from other pathways (Appleby *et al*., 1996). A large majority of HPt domains are part of multi-domain HKs containing a histidine residue capable of participating in phosphoryl transfer reactions. HPt, which functions as a receiver and donor shuttling phosphoryl groups between two or more RR domains, is suggested to connect different TCSs in a specific fashion (Perraud *et al*., 1999). HPt domains provide additional points for regulation in the TCS signalling pathway.

2.3. Response regulator

Response regulators contain a regulatory domain of approximately 120 residues that is phosphorylated via Mg^{2+}-dependent reaction by a histidine kinase (Goudreau and Stock, 1998). The important residues for phosphorylation are a pair of aspartates near the N-terminus, a centrally located aspartate, which is a site of phosphorylation, and a lysine near the C-terminus (Parkinson, 1993). Furthermore, as many HKs have specific RR phosphatase activity, RRs themselves typically have autophosphatase activity that limits the lifetime of the phosphorylated state, yielding half-lives in the range of seconds to hours (West and Stock, 2001). RR proteins usually have two domain structures: a conserved N-terminal regulatory domain, also referred to as a receiver domain, and a variable C-terminal effector domain, also called an output domain (Parkinson, 1993; West and Stock, 2001). Most cytoplasmic RRs have a DNA-binding output domain, which controls the expression of one or several target genes at the transcriptional level (Parkinson, 1993). RRs are divided into three major subfamilies based on sequence similarities between their C-terminal output domains: OmpR/PhoB winged helix domains, NarL/FixJ four-helix domains and NtrC ATPase-coupled transcription factors (Stock et al., 1989; West and Stock, 2001).

3. Regulation of virulence in *Ecc*

Bacteria regulate gene expression by diverse mechanisms. Virulence gene regulation involves complex regulatory circuits that respond to environmental cues. These regulatory components include two-component systems, sigma factors, quorum-sensing systems and regulatory RNAs (Cotter and Miller, 1998; Storz and Hengge-Aronis, 2000).

3.1. ExpS-ExpA two-component system

The transposon mutagenesis of the *Ecc* wild-type strain SCC3193 led to the discovery of a legion of mutants that fell into two broad categories: motility mutants showing a decreased virulence (Mot^-) and avirulent mutants exhibiting decreased production of extracellular enzymes (Exp^-) (Pirhonen *et al*., 1991). One transposon insertion was localized in the *expA* gene encoding a response regulator that resulted in an avirulent phenotype and showed decreased production of extracellular enzymes cellulase, polygalacturonase and pectate lyases (Eriksson *et al*., 1998). Furthermore, ExpA activated expression of the *prtW* gene encoding a protease (Marits *et al*., 1999; 2002). Another mutant was shown to contain an insertion in the *expS* gene encoding a sensor histidine kinase (Eriksson *et al*., 1998). Inactivation of the *expS* decreased production of extracellular enzymes, although not as drastically as observed in the *expA* mutant strain. The potato maceration capacity was highly reduced in the *expA* mutant, whereas the *expS* mutant retained 70% of its maceration capacity. On the basis of structural similarities with known TCSs in other bacterial species, the *expA* and *expS* gene products were suggested to form a two-component system (TCS) (Eriksson *et al*., 1998; Figure 2).

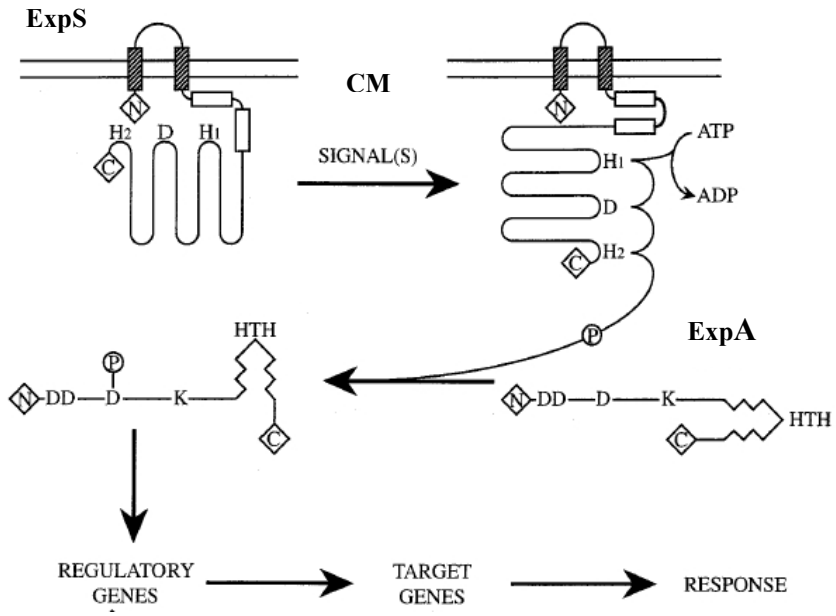


Figure 2. Model of the signal transduction pathway mediated by the ExpS-ExpA two-component system (adapted and modified from Heeb and Haas, 2001). Conserved aspartate (D), histidine (H) and lysine (K) residues; CM, cytoplasmic membrane; HTH, helix-turn-helix motif; N, N-terminus; C, C-terminus.

Frederick *et al*. (1997) described the ExpS homologue RpfA from another *Ecc* strain, AH2. $RpfA_{AH2}$ was shown to be responsible for activation of cellulase and protease but not polygalacturonase or pectate lyase. In addition, $GacA_{Ecc71}$ (ExpA homologue) was verified to activate expression of $hrpN_{ECC71}$ along with extracellular enzyme genes (Cui *et al*., 2001). In contrast to the results of Frederick *et al*. (1997), the $rpfA_{AH2}$ mutant (renamed *gacS)* also showed decreased expression of *pel* and *peh* gene products (Cui *et al*., 2001). The $GacA_{Ecc71}$ protein was highly homologous to $ExpA_{SCC3193}$, with 93% identity at the amino acid level (Table 2). Furthermore, the $gacS_{Ecc71}$ gene product had 93% and 91% identity to $RpfA_{AH2}$ and $ExpS_{SCC3193}$, respectively (Cui *et al*., 2001; Table 3).

3.1.1. ExpA response regulator

The *expA* gene consists of 654 nucleotides and encodes a cytoplasmic 218-aa polypeptide with a calculated molecular weight of 24 kDa (Eriksson *et al*., 1998). This gene is organized in the same operon with a second downstream gene, *uvrC*, which encodes a protein involved in UV repair system. The organization of *expA* and *uvrC* genes is conserved in enterobacterial genomes. Inactivation of the *expA* as well as its homologues has resulted in reduced UV tolerance, indicating that these genes are cotranscribed (Eriksson *et al*., 1998; Heeb and Haas, 2001). The ExpA polypeptide contains a typical N-terminal receiver domain of a response regulator and a C-terminal helix-turn-helix (HTH) DNA-binding domain. ExpA has been classified into the FixJ family of RRs (Eriksson *et al*., 1998), which belongs to the LuxR superfamily of bacterial regulatory proteins.

3.1.2. ExpA orthologues in the *Enterobacteriaceae* family

The ExpA orthologues in other bacterial species are shown in Table 2. Interestingly, recent genome sequence of *Erwinia carotovora* subsp. *atroseptica* (*Eca*; Bell *et al*., 2004) revealed that $ExpA_{SCC3193}$ is 98% identical to the *Eca* ExpA protein, and this identity is higher than that reported for $GacA_{Ecc71}$ (Cui *et al.*, 2001).

In other enterobacterial species, the highest identity of ExpA orthologues with characterized function was observed in *Serratia plymuthica* (Table 2). This plant-associated strain has been described as a biocontrol strain, a wide-range antagonist of many microorganisms, including pathogenic fungi (Ovadis *et al*., 2004). The *grrA* mutant strain was deficient in production of antifungal compounds, such as the antibiotic pyrrolnitrin, exoprotease and the ChiA endochitinase, as well as in antifungal activity against *Rhizoctonia solani* and *Pythium aphanidermatum* (Ovadis *et al*., 2004). In addition, production of the quorum sensing signal (*N*-acyl-homoserine lactone, AHL) was down-regulated in the *grrA* mutant compared with the wild-type strain (Ovadis *et al*., 2004).

In *Salmonella enterica* serovar Typhimurium, the ExpA orthologue SirA positively regulates the type III secretion system, bacterial invasion of epithelia and elicitation of bovine gastroenteritis (Johnston *et al*., 1996; Ahmer *et al*., 1999; Goodier and Ahmer, 2001). SirA is encoded outside of SPI1, and it positively regulates another regulatory gene, *hilA* (Bajaj *et al*., 1996), encoded within SPI1, which in turn activates the genes encoding the structural components of the SPI1 type III secretion system. SirA was shown to negatively affect motility in *Salmonella* by decreasing expression of *flhDC* genes, which encode the master

regulon of flagellar genes (Goodier and Ahmer, 2001). Furthermore, SirA-dependent regulation of motility and virulence was suggested to be mediated through independent pathways (Teplitski *et al*., 2003; Figure 3).

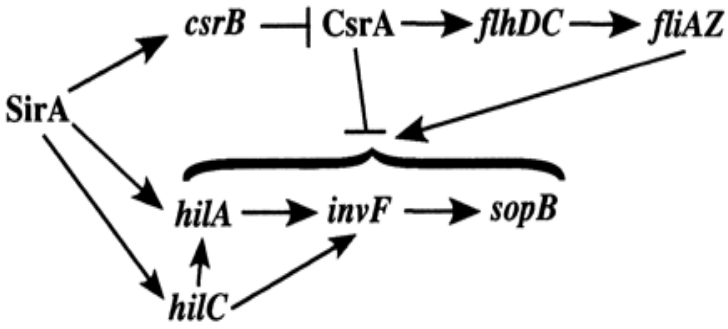


Figure 3. Model of the SirA regulatory cascade in *Salmonella* (adapted from Teplitski *et al*., 2003). SirA directly activates the *csrB*, *hilA*, and *hilC* promoters. The *csrB* RNA directly binds and antagonize the activity of CsrA. Thus SirA activates virulence and repress motility independently although these two regulatory cascades interact with each others.

The ExpA orthologue UvrY of *E. coli* K-12 (Sharma *et al*., 1986) showed 99% identity at the amino acid level to the UvrY proteins of pathogenic *E. coli* strains (e.g. O6, CFT073, O157:H7) and 96% identity to the SirA of *S. typhimurium*. Most of the studies characterizing $UvrY_{E.coli}$ have been done with *E. coli* K-12. The function of UvrY in pathogenic strains is based on sequence similarity, although some extensive genomic variation has been observed by comparing pathogenic strains with each other and with non-pathogenic strains (Welch *et al*., 2002). In *E. coli* K-12, inactivation of the *uvrY* gene led to a phenotype hypersensitive to hydrogen peroxide, suggesting that UvrY is necessary to protect against hydrogen peroxide-mediated stress due to reduced expression of catalase (Pernestig *et al*., 2001). UvrY was also shown to activate biofilm formation (Suzuki *et al*., 2002). Furthermore, UvrY was suggested to be crucial for efficient adaptations between metabolic pathways (Pernestig *et al*., 2003). The *uvrY* mutant had a growth advantage with gluconeogenic carbon sources when competing with the wild type, but carbon sources feeding into glycolysis led to the opposite result. These long-term competition studies were done with both uropathogenic and non-pathogenic *E. coli* strains (Pernestig *et al*., 2003). ExpA orthologues in the *Vibrionaceae* and *Pseudomonaceae* families are presented together with their main characteristics in Table 2. The evolutionary rate for the *expA* gene has been suggested to be higher in *Enterobacteriaceae* than in *Pseudomonaceae* (de Souza *et al*., 2003). The ExpA orthologues are evidently responsible for global regulatory circuits in various γ-proteobacteria.

Table 2. ExpA orthologues and their function in γ-proteobacterial species.

Species	Protein	Identity/ similarity %[a]	Relevant characteristics	Reference
Erwinia carotovora ssp. *carotovora*	GacA	93/-[b]	activates production of PCWDEs and expression of *hrpN* and *rsmB* RNA	Cui *et al*. (2001)[c]
Serratia plymuthica	GrrA	85/93	positive control of pyrrolnitrin, exoprotease, AHL and endochitinase	Ovadis *et al*. (2004)[c]
Salmonella typhimurium	SirA	83/92	negative control of FlhDC operon through *csrB*, positive control of *hilA* that mediates regulation of SPI1 and other virulence factors	Johnston *et al*. (1996); Goodier and Ahmer (2001); Teplitski *et al*. (2003)
Escherichia coli K-12	UvrY	82/92	controls glycolysis and gluconeogesis through CsrA/*csrB*; necessary to protect against H_2O_2	Pernestig *et al*. (2001)[c]; Pernestig *et al*. (2003)[c]
Vibrio fischeri	GacA	73/87	activates symbiosis, e.g. luminescence, production of siderophores, motility	Whistler and Ruby (2003)
cholerae	VarA	72/87	activates cholera toxin production and toxin-coregulated pilus	Wong *et al*. (1998)
Pseudomonas fluorescens	GacA	59/76	controls production, e.g. pyoluteorin, pyrrolnitrin,	Laville *et al*. (1992); Blumer *et al*. (1999)[c]
aeruginosa	GacA	59/76	hydrogen cyanide, protease, AHL. Reduced biocontrol	Aarons *et al*. (2000); Reimmann *et al*. (1997);
syringae	GacA	58/75	activity in suppression of plant diseases, reduced	Rich *et al*. (1994)[c]
putida	GacA	48/62	ecological fitness and survival in stationary phase[d]	Bertani and Venturi (2004)

[a] compared with $ExpA_{SCC3193}$

[b] sequences of *gacA*/*gacS* of *Ecc* strain 71 were unavailable

[c] similar characteristics have been described for cognate sensor kinase protein; see Table 3

[d] features for plant-associated strains

Table 3. ExpS orthologues and their function in γ-proteobacterial species.

Species	Protein	Identity/ similarity %[a]	Relevant characteristics	Reference
Erwinia carotovora ssp. *carotovora*	RpfA	92/94	positive control of cellulase and protease activation of *pel-1* and *peh-1* expression	Frederick *et al*. (1997) Cui *et al*. (2001)
Serratia plymuthica	GrrS	64/76	positive control of pyrrolnitrin, exoprotease, AHL and endochitinase	Ovadis *et al*. (2004)
Salmonella typhimurium	BarA	61/75	activation of invasion genes, acetate restored *barA* phenotype, not required for virulence	Altier *et al.* (2000) Lawhon *et al*. (2002)
Escherichia coli K-12	BarA	61/73	multicopy suppressor of *envZ* mutation, transcriptional activation of *rpoS*	Nagasawa *et al*. (1992) Mukhopadhyay *et al*. (2000)
DS17	AirS	61/73	activation of siderophore production	Zhang and Normark (1996)
Pseudomonas syringae	LemA	37/54		Hrabak *et al*. (1992); Rich *et al*. (1994)
sp. PCL1171	GacS	37/53	role in colony phase variation	Kitten *et al*. (1998); van den Broek *et al*. (2003)

[a] compared with $ExpS_{SCC3193}$

3.1.3. ExpS histidine kinase

The *expS* gene encodes a 928-aa polypeptide with a calculated molecular mass of 104.2 kDa (Eriksson *et al*., 1998). ExpS contains two transmembrane regions and is most likely associated with the cytoplasmic membrane (Figure 2). It is related to a number of unorthodox types of histidine kinases with linked transmitter and receiver modules. ExpS orthologues have been characterized in several different bacterial species, but the signal activating this histidine kinase is still unknown (Table 3).

In a recent review, BarA of *E. coli* was proposed to function as a global response regulator as well (Sahu *et al*., 2003). Its role in oxidative, weak acid and osmotic stress responses and in biofilm formation was supported by preliminary experimental DNA microarray data showing its involvement in cell division, carbon metabolism, iron metabolism and pili formation (Sahu *et al*., 2003). The complex control of several stress responses by $BarA_{E.coli}$ was demonstrated to be related to the control of the secondary sigma factor RpoS (Mukhopadhyay *et al*., 2000). The hydrogen peroxide sensitivity of a $barA_{E.coli}$ mutant could be explained by a loss of RpoS-dependent catalase activity and BarA being required for transcriptional induction of RpoS. However, the results of *rpoS* transcriptional regulation are controversial in different species, and studies of its regulation have revealed one of the most complex regulatory mechanisms in bacteria (Venturi, 2003).

The characteristics of *Salmonella* BarA have mostly been based on studies of *E. coli* since these proteins share 90% identity with each other. Interestingly, the inactivation of *Salmonella barA* was not found to have a significant effect on LD_{50} in mice (Lawhon *et al*., 2002). In addition, acetate restored the expression of invasion genes in the *barA* mutant, but had no effect on the *sirA* mutant. The authors suggested that in the mouse intestinal tract two independent signals exist for the expression of SPI1 genes, acetate being one and the other unknown substance, which act through BarA of *Salmonella*.

3.2. RsmC-RsmA-*rsmB* RNA regulatory system

3.2.1. Global repressor RsmA

Transposon mutagenesis of strain Ecc_{Ecc71} resulted in isolation of a $RsmA^-$ mutant which overproduced the extracellular enzymes pectate lyases, polygalacturonase and cellulases (Chatterjee *et al*., 1995b). The $rsmA^+$ locus contained a 183-bp open reading frame (ORF) encoding a 6.8-kDa RsmA protein (Cui *et al*., 1995) 95% identical to the central carbohydrate metabolism regulator CsrA of *E. coli* (Romeo *et al*., 1993). Overexpression of the $rsmA_{Ecc71}$ gene suppressed the expression of extracellular enzymes, plant tissue maceration and elicitation of hypersensitive reaction in tobacco leaves (Cui *et al*., 1995; 1996). The presence of the *rsmA* gene was verified in various *Erwinia* strains. Overproduced $RsmA_{Ecc71}$ decreased diverse phenotypical traits such as flagellum formation and motility in $Ecc_{SCC3193}$ and production of extracellular polysaccharides and pathogenicity in other *Erwinia* strains (Cui *et al*., 1995; Mukherjee *et al*., 1996).

3.2.2. Regulatory RNA *rsmB*

The *rsmB* (formerly *aepH*) gene was originally suggested to encode a small 5.45-kDa highly basic protein that activated the production of extracellular enzymes in Ecc_{Ecc71} (Murata *et al*., 1994). The DNA segment containing the $rsmB_{Ecc71}$ gene activated the production of polygalacturonase, pectate lyases and cellulases even in *E. coli* carrying the cognate genes (Murata *et al*., 1994). Further characterization of the *rsmB* locus confirmed that expression of *rsmB* resulted in production of two RNA species, a 479-base primary *rsmB* RNA which was processed to yield a 259-base *rsmB*' RNA (Liu *et al*., 1998). Interaction between the RNA-binding protein RsmA and RNA repeats in the 5' region of the primary *rsmB* transcript was suggested based on studies with the *E. coli* CsrA-*csrB* regulatory system (Liu MY *et al*., 1997; Liu *et al*., 1998). The processed *rsmB*' RNA was proposed to mediate its regulatory effect by neutralizing the RsmA repressor, which in turn affects positively on expression of the target genes (Liu *et al*., 1998). Furthermore, the *rsmB* RNA was more stable in the presence of functional RsmA, suggesting a role for RsmA in modulation of *rsmB* RNA levels (Chatterjee *et al*., 2002a).

Although the overall nucleotide homology between *rsmB* and *csrB* of *E. coli* was low, expression of *rsmB* in *E. coli* suppressed negative effects of CsrA on glycogen accumulation, implying that these RNAs are functionally similar (Liu MY *et al*., 1997). The existence of the *rsmB* gene was verified by Southern blot analysis in various *Ecc* and *Eca* strains. It is also present in other *Erwinia* and enterobacterial species, suggesting that these bacteria employ the RsmA-*rsmB* regulatory system to modulate their gene expression (Murata *et al*., 1994; Ma *et al*., 2001). However, the RsmA-*rsmB* system is supposed to affect a different set of genes in animal and plant pathogens (Chatterjee *et al*., 2002a). The TCS GacS-GacA (ExpS-ExpA homologues) has been shown to activate expression of *rsmB* RNA in Ecc_{71} (Cui *et al*., 2001). The GacS-GacA was suggested to act via the RsmA-*rsmB* regulatory system to control the production of extracellular enzymes and *hrp* genes.

3.2.3. Global regulator RsmC

A novel type of virulence regulatory gene *rsmC* (also known as *hexY*) encoding a 14.5-kDa cytoplasmic protein negatively controls virulence in *Ecc* (Cui *et al*., 1999; Shih *et al*., 1999). A mutation in the *rsmC* gene increased the expression of *pel-1*, *peh-1*, *celV* and *hrpN* genes, the production of proteases and the maceration capacity of celery petioles in Ecc_{Ecc71} (Cui *et al*., 1999). Furthermore, inactivation of $rsmC_{MH1000}$ caused increased production of pectate lyases, cellulases and proteases and increased potato maceration capacity but clearly decreased the bacterial growth rate (Shih *et al*., 1999). The RsmC protein has no known homologues among prokaryotes and was suggested to be a regulator specific to soft-rot *Erwinia carotovora* subspecies (Cui *et al*., 1999; Shih *et al*., 1999). $RsmC_{Ecc71}$ was shown to control *rsmA* negatively and *rsmB* positively at the transcriptional level, although the exact mechanism remains unsolved (Cui *et al*., 1999). The $rsmC_{MH1000}$ gene was found to contain an unusually long 5' untranslated region suggested to be involved in post-transcriptional regulation (Shih *et al*., 1999). Inactivation of $rsmC_{Ecc71}$ did not affect levels of global regulator genes *kdgR*, *hexA*, *rpoS*, *hor* or *hslI* (Cui *et al*., 1999).

3.3. KdgR repressor, a member of the IclR family

The *kdgR* gene was characterized from two different *Ecc* strains (Liu *et al*., 1999; Thomson *et al*., 1999). KdgR had formerly been shown to repress the production of pectinolytic enzymes and secretion of pectinase in *Echr* (Hugouvieux-Cotte-Pattat *et al*., 1996). It was also found to negatively control the expression of *celV* and *hrpN* genes and the production of protease in Ecc_{Ecc71} (Liu *et al*., 1999). The binding of KdgR to the *pel-1* and *peh-1* promoter regions was confirmed with gel mobility shift assays, indicating that KdgR interferes with the initiation of transcription, as previously shown for some of the *Echr pel* genes (Liu *et al*., 1999). The KdgR homologues shared 92.8% amino acid identities between Ecc_{EC1} and $Echr_{3937}$, including a complete match of the hypothetical DNA-binding (HTH) motif. However, the lower binding efficiency of $KdgR_{Echr}$ to the promoter regions of the pel_{Ecc} and peh_{Ecc} genes suggests differences in its binding regions on these genes (Matsumoto *et al*., 2003a).

Furthermore, KdgR bound to three KDGR boxes located within the transcriptional unit of the *rsmB* gene (Liu *et al*., 1999; see Figure 4). However, the KdgR binding site was located 79 bases downstream of the transcriptional initiation site of *rsmB*, suggesting that KdgR may not interfere with initiation of transcription but instead may affect elongation of transcription (Liu *et al*., 1999). The authors proposed a putative model showing KdgR and RsmA-*rsmB* RNA-mediated regulatory effects (Liu *et al*., 1999; Figure 4). The negative effect of $KdgR_{Ecc71}$ on expression of *celV*, *prt* and *hrpN* genes was proposed to be mediated through *rsmB* expression (Liu *et al*., 1999).

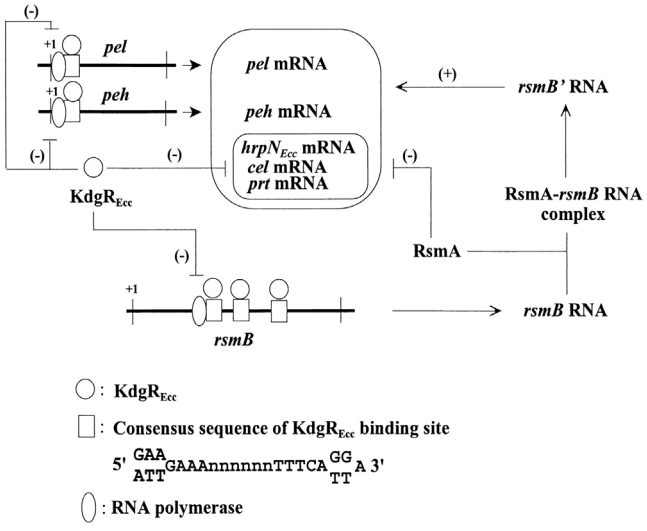


Figure 4. A speculative model depicting the regulatory effects of $KdgR_{Ecc71}$ on the production of extracellular enzymes and $Harpin_{Ecc71}$ (adapted from Liu *et al*., 1999).

Along with the KdgR repressor Thomson *et al*. (1999) identified the RexZ protein, another member of the IclR family of DNA-binding proteins, but despite sharing some sequence identity with KdgR these two proteins were not functional homologues. The RexZ protein was shown to activate the production of extracellular enzymes, thereby affecting

virulence (Thomson *et al*., 1999). In addition, the results suggested that the RexZ protein was controlled directly by the cAMP-CRP complex, and not by the KdgR repressor.

3.4. Other regulatory proteins

Other regulators affecting virulence in *Ecc* are presented in Table 4. However, as there are many global and gene-specific systems controlling extracellular enzyme expression and virulence, the function and putative hierarchy of proteins with respect to each other are only partially unravelled.

3.4.1. PehR-PehS two-component system

A specific activator of the $pehA_{SCC3193}$ gene encoding polygalacturonase was shown to be a TCS PehR-PehS that responded to extracytoplasmic calcium and magnesium levels (Saarilahti *et al*., 1992; Flego *et al.*, 1997, 2000). Inactivation of the *pehR* gene encoding a response regulator resulted in impaired production of polygalacturonase and reduced virulence in tobacco seedlings, but the production of pectate lyases and cellulases was as in the wild type (Saarilahti *et al*., 1992). The mutated *pehS* gene encoding a cognate sensor kinase resulted in a similar phenotype as that observed for the *pehR* mutant strain, and the *pehR-pehS* operon was verified to encode a TCS highly similar to the PhoP-PhoQ of *E. coli* and *S. enterica* serovar Typhimurium (Flego *et al*., 2000; Figure 1). An intact *phoP* gene was required for expression of *pehA* in *E. coli*, where *pehA* was also repressed in a Ca^{2+}-dependent manner. Furthermore, the expression of *pehRS* genes restored the non-specific acid phosphatase-negative phenotypes of *Salmonella phoP* and *phoQ* mutants, suggesting that these TCSs are functionally similar (Flego *et al*., 2000). The PhoP-PhoQ TCS has been considered a major virulence regulatory system controlling numerous different genes in *S. typhimurium* (Groisman, 2001).

3.4.2. AepA activator protein

The *aepA* gene was identified to encode a potential membrane-associated protein activating the production of extracellular enzymes in Ecc_{71} (Murata *et al*., 1991; Liu *et al*., 1993). The expression of the *aepA–lacZ* operon fusion was constitutive in *E. coli* but inducible by plant extracts in *Ecc*, suggesting that *aepA* may be under negative control in *Ecc* (Liu *et al*., 1993). The *aepA* gene was identified by Southern blot in several *Ecc* strains; however, the AepA protein showed no significant homology to known prokaryotic regulatory proteins (Liu *et al*., 1993); nor did an updated search from sequence databanks uncover any homologies. Although the AepA protein might be restricted to *Erwinia* species, its N-terminus contains a domain of metallo-dependent hydrolases found in many bacterial and fungal enzymes.

Table 4. Other regulators affecting virulence in *Ecc*.

Protein/system	Relevant characteristics	Mechanism	Reference
PehS-PehR[1]	TCS that activates Peh production and responds to extracellular Ca^{2+} and Mg^{2+} levels	unknown	Flego *et al*. (2000)
AepA[1]	activates production of PCWDEs	unknown	Murata *et al*. (1991); Liu *et al*. (1993)
HexA[1]	negative regulation of PCDWEs, $Harpin_{Ecc71}$, AHL, *rsmB* RNA and RpoS	DNA-binding (*pelC*)	Harris *et al*. (1998); Mukherjee *et al.* (2000)
ExpM[1]	negative regulation of RpoS, activates virulence	decreases RpoS stability	Andersson *et al*. (1999b)
RpoS[1]	against several stress responses in stationary phase; inactivation increases virulence	sigma-S	Mukherjee *et al.* (1998); Andersson *et al*. (1999a)
ExpI-ExpR[1]	N-acyl-homoserine lactone (AHL) synthesis, activation of PCDWEs and virulence	unknown	Jones *et al*. (1993); Pirhonen *et al*. (1993)
CytR[3]	positive control of Peh production, flagellum synthesis and virulence	unknown	Matsumoto *et al*. (2003b)
Hor[2]	activates production of PCDWEs and ß-lactam antibiotic carbapenem	unknown	Thomson *et al*. (1997)
RdgA-RdgB[3]	transcriptional activator of *pnlA* gene	DNA-binding	Liu Y. *et al*. (1997)

[1] identified in *Ecc* strain SCC3193

[2] absent from *Ecc* strain SCC3193

[3] not identified in *Ecc* strain SCC3193

3.4.3 HexA-ExpM-RpoS regulatory cascade

3.4.3.1. HexA repressor

The HexA regulator negatively controls extracellular enzyme production in both Ecc_{MH1000} and $Eca_{Em56.2}$, as well as motility in $Eca_{Em56.2}$ (Harris *et al*., 1998). The *hexA* gene encodes a close homologue of the $pecT_{Echr}$ gene product, which is a member of the LysR family of transcriptional regulators containing conserved DNA-binding domains (Hugouvieux-Cotte-Pattat *et al*., 1996). Inactivation of *hexA* increased production of cellulase, pectate lyase and protease but had no obvious effect on polygalacturonase production (Harris *et al*., 1998). Expression of the *hexA* gene from a low-copy plasmid caused a more than 4-fold reduction in the expression of *pelC-lacZ* and *celV-lacZ* fusions, and the HexA protein was shown to bind to the *pelC* promoter in a gel-shift mobility assay (Harris *et al*., 1998). Furthermore, because the *hexA* mutant produced higher levels of AHL, especially during the early logarithmic growth phase, the authors proposed that HexA could be an activator of *rsmA* transcription (Harris *et al*., 1998).

However, in the other *Ecc* strain, $HexA_{Ecc71}$ also repressed production of polygalacturonase, while accumulation of AHL remained dependent on cell density, suggesting that the effect of HexA may vary to some extent depending upon the *Ecc* strain (Mukherjee *et al*., 2000). In addition, $HexA_{Ecc71}$ negatively regulated expression of the $hrpN_{Ecc71}$ and transcription of the *rsmB* encoding the regulatory RNA. Presence of the *hexA* homologues was confirmed by Southern blot in various *Erwinia* species (Mukherjee *et al*., 2000). A speculative model was proposed where HexA regulates RpoS stability by ExpM and ClpX/ClpP components, and RpoS, in turn, affects the production of virulence determinants by controlling expression of *rsmA* (Mukherjee *et al*., 2000; Figure 5).

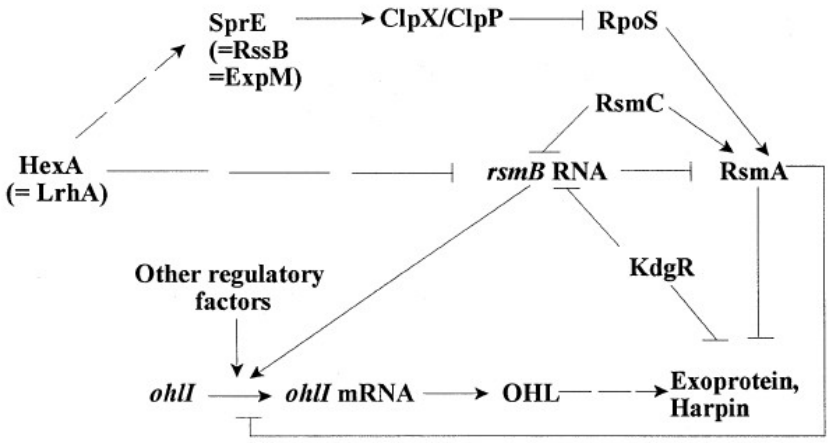


Figure 5. A speculative model depicting HexA-dependent major regulatory events controlling the production of extracellular enzymes, AHL and $Harpin_{Ecc71}$ in Ecc_{Ecc71} (adapted from Mukherjee *et al*., 2000).

3.4.3.1. ExpM response regulator

Transposon mutagenesis carried out by Pirhonen *et al*. (1991) resulted in isolation of a pectin lyase-negative mutant characterized to contain an insertion in the e*xpM* gene encoding a response regulator (Andersson *et al*., 1999b). ExpM affected production and secretion of extracellular enzymes and activated virulence *in planta*. The results suggest that $ExpM_{SCC3193}$ is a functional homologue of the response regulators RssB (SprE) of *E. coli* and MviA of *S. typhimurium*, which both lack a typical DNA-binding domain and have been shown to negatively regulate the stability of the stationary phase sigma factor RpoS (Andersson *et al*., 1999b; Hengge-Aronis, 2000; see Figure 5). In addition, Mukherjee *et al*. (2000) confirmed that an *expM*-like gene is present in most *Erwinia* and many other enterobacterial species.

3.4.3.2. Sigma factor RpoS

The RpoS is a transcription factor and regulator for a number of genes activated during the stationary phase and required for survival upon nutrient limitation or stress, e.g. acidic pH (Venturi, 2003). Inactivation of $rpoS_{Ecc71}$ increased sensitivity to hydrogen peroxide, carbon starvation and acidic pH, indicating a function similar to that of $RpoS_{E.\ coli}$ (Mukherjee *et al*., 1998). The $rpoS_{Ecc71}$ mutant produced higher levels of polygalacturonase, pectate lyases and cellulase, showed increased expression of $hrpN_{Ecc71}$ and caused more extensive maceration on celery petioles than the $RpoS^+$ strain (Mukherjee *et al*., 1998). These effects were suggested to be mediated through the control of *rsmA* expression (see Figure 5).

The $rpoS_{SCC3193}$ mutant also exhibited increased sensitivity to osmotic and oxidative stress and deficiency in glycogen accumulation (Andersson *et al*., 1999a). The production of extracellular enzymes was similar *in vitro* in the *rpoS* mutant and in the wild type. However, the *rpoS* mutant produced more extracellular enzymes and especially necrosis-inducing protein $Nip_{SCC3193}$ *in planta* (Andersson *et al*., 1999a; Mattinen *et al*., 2004). The $rpoS_{SCC3193}$ mutant showed increased sensitivity in transgenic tobacco plants, exhibiting higher levels of reactive oxygen species. In addition, $RpoS_{SCC3193}$ was also a positive regulator of *rsmA* expression. The results suggest that RpoS is necessary mainly for survival in a competitive environment (Andersson *et al*., 1999a).

3.4.4. ExpI-ExpR quorum sensing system

Bacterial cell-to-cell signalling, an intercellular mechanism referred to as quorum sensing, allows bacteria to sense, integrate and process information from their surroundings (Hardman *et al*., 1998; Pierson *et al.,* 1998; Fuqua and Greenberg, 2002; Whitehead *et al*., 2002; Newton and Fray, 2004). The production and utilization of diffusible *N*-acyl-homoserine lactones (AHLs) provide a means for bacteria to regulate the expression of specific genes and operons e.g. luminescence, competence or virulence (Fuqua and Greenberg, 2002).

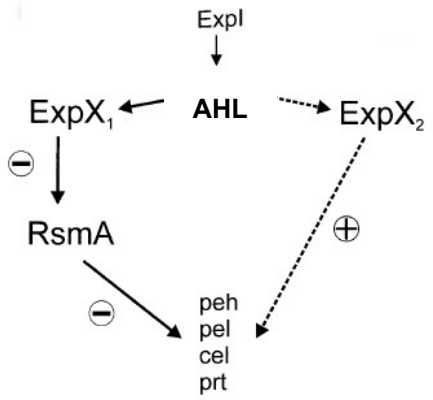


Figure 6. A speculative model depicting the regulatory effect of AHL on the production of the extracellular enzyme in $Ecc_{SCC3193}$ (adapted from Kõiv and Mäe, 2001).

One avirulent Exp$^-$ mutant (Pirhonen *et al*., 1991) was found to contain a mutation in the $expI_{SCC3193}$ (also known as $hslI_{Ecc71}$/$ohlI_{Ecc71}$) gene encoding the AHL synthetase. This regulatory system, quorum sensing was shown to activate the production of polygalacturonase, pectate lyase, cellulase and protease (Pirhonen *et al*., 1993; Chatterjee *et al*., 1995b; Marits *et al*., 1999). In addition, inactivation of *carI* (*expI* homologue) in another *Ecc* strain resulted in deficiency in carbapenem antibiotic production (Bainton *et al*., 1992; Jones *et al*., 1993; Swift *et al*., 1993). However, the LuxR homologues CarR and ExpR do not appear to be AHL-responsive transcriptional activators for the expression of extracellular enzymes in *Ecc* (Salmond *et al*., 1995; Holden *et al*., 1998; Andersson *et al*., 2000), although binding was confirmed between AHL and CarR in activation of the *car* gene cluster (Welch *et al*., 2000).

Earlier studies have suggested that the global repressor RsmA negatively controls quorum sensing (*ohlI*) in Ecc_{Ecc71} (Chatterjee *et al*., 1995b; Cui *et al*., 1995; see Figure 5). Subsequent research has, however, revealed that quorum sensing signal AHL itself was affecting expression of $rsmA_{SCC3193}$ (Kõiv and Mäe, 2001; Figure 6) and that $RsmA_{Ecc71}$ also influenced the stability of RsmB RNA (Chatterjee *et al*., 2002a). These results imply that the quorum sensing signal is channelled via the RsmA-*rsmB* RNA post-transcriptional regulatory system (Kõiv and Mäe, 2001; Chatterjee *et al*., 2002a).

3.4.5. CytR, a member of the LacI family

The CytR protein was shown to positively control polygalacturonase production and flagellum synthesis and to play an important role during pathogenesis in Ecc_{EC1} (Matsumoto *et al*., 2003b). CytR was suggested to modulate the flagellum master regulon FlhDC at post-transcriptional or post-translational levels. The authors suggested that this control could involve one or more molecular chaperons or one or more proteases, e.g. ClpX/ClpP, or even both (Matsumoto *et al*., 2003b). A *cytR* homologue was also found to exist in other enteric bacteria, like *E. coli* and *Salmonella*, but not in genera such as *Pseudomonas*, *Ralstonia* or *Agrobacterium* (Matsumoto *et al*., 2003b). In *E. coli*, the CytR represses genes responsible for nucleoside uptake and catabolism in nucleoside-poor environments.

3.4.6. Hor protein, a member of the SlyA family

A *hor* gene product activated the production of β-lactam antibiotic carbapenem and extracellular enzymes in Ecc_{GS101} (Thomson *et al*., 1997). The *hor* gene was verified by Southern blot analysis in several *Ecc* strains but not in carbapenem-negative $Ecc_{SCC3193}$ (Thomson *et al*., 1997; Holden *et al*., 1998). The Hor protein is a SlyA homologue earlier suggested to be restricted to enteroinvasive *E. coli*, *Shigella* and *Salmonella* species. The SlyA DNA-binding protein is responsible for controlling diverse physiological processes in these pathogenic enterobacterial species.

3.4.7. RdgA-RdgB regulatory system

A specific activator RdgB for the *pnlA* gene encoding pectin lyase was identified in the Ecc_{Ecc71} (Liu Y *et al*., 1997). DNA-damaging agents, such as mitomycin C, nalidixic acid and UV light, induced the *rdg* locus controlling the expression of *pnlA* gene (McEvoy *et al*., 1992). The transcription of *rdgA* was autoregulated and the RecA-processed RdgA protein derepressed the expression of *rdgB* (Liu Y *et al*., 1994b, 1996, 1997; Figure 7). The RdgB bound specifically to the *pnlA* promoter and a putative KdgR-binding site was localized immediately downstream, suggesting that the KdgR repressor could block *pnlA* transcription under normal growth conditions, i.e. in the absence of RdgB (Liu Y *et al*., 1997). In addition, the RdgB was suggested to regulate genes involved in synthesis of the bacteriocin carotovoricin and cellular lysis (Liu Y *et al*., 1997).

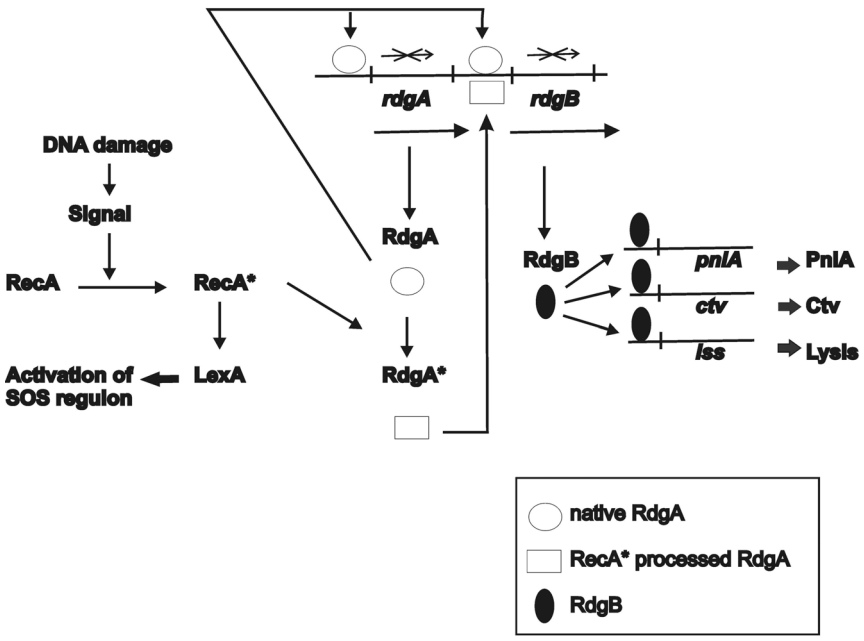


Figure 7. A tentative model depicting the regulation of pectin lyase (Pnl) production and other damage-induced traits in Ecc_{Ecc71} (adapted from Liu *et al*., 1994).

B. AIMS OF THE STUDY

The two-component system (TCS) ExpS-ExpA is a global signal transduction system characterized from a number of Gram-negative bacteria. It appears to be one of the most important regulatory systems activating virulence gene expression in pathogens. Despite extensive research, the signal(s) activating this TCS remains obscure. Furthermore, the mechanism by which the message is delivered downstream to the target genes is unknown. At the beginning of this study, the ExpS-ExpA had already been characterized as an important regulatory system activating the production of extracellular enzymes in *Erwinia carotovora* ssp. *carotovora*. The first hypothesis suggested direct regulation of extracellular enzyme genes by ExpA. The aim here was to characterize regulatory components acting in concert with ExpA or serving as downstream regulators of ExpA in the activation process of virulence genes.

C. MATERIALS AND METHODS

The bacterial strains and plasmids in studies I-III are listed in Tables 5 and 6, respectively. Construction of mutant strains, growth conditions and plasmid constructions have been described in the original publications. The methods used are listed in Table 7; more detailed information can be found in the original articles.

Table 5. Bacterial strains used in studies I-III.

Strain	Genotype or relevant characteristics	Reference/source
E. coli		
HB101	F$^-$ *thi-1 hsdS20* (r$^-$, m$^-$) *supE44 recA13 ara-14 leuB6 proA2 lacY1 rpsL20* (Str^R) *xyl-5 mtl-1*	Boyer and Roulland-Dussoix (1969)
S17-1 λ pir	Tp^R Sm^R *recA thi pro hsdR$^-$M$^+$* RP4:2-Tc: Mu: Km Tn7 λ*pir*	Miller and Mekalanos (1988)
TG1	Δ(*lac-pro*) *supE thi hsd5* (F' *traD36 proA$^+$B$^+$ lacI^q lacZ*Δ*M15*)	Carter *et al*. (1985)
JM109	e14$^-$(McrA-) *recA1 endA1 gyrA96 thi-1 hsdR17*($r_{K-}m_{K+}$) *supE44 relA1* Δ(*lac-proAB*) [F' *traD36 proAB lacI^{q}Z*Δ*M15*]	Yanish-Perron *et al*. (1985)
JM110	*rpsL* (Strr) *thr leu thi-1 lacY galK galT ara tonA tsx dam dcm supE44* Δ(*lac-proAB*) [F' *traD36 proAB lacI^{q}Z*Δ*M15*]	Yanish-Perron *et al*. (1985)
XL1-Blue MRA(P2)	Δ(*mcrA*)183Δ(*mcrCB-hsdSMR-mrr*)173 *endA1 supE44 thi-1 gyrA96 relA1 lac* (P2 lysogen)	Stratagene
E. carotovora ssp. *carotovora*		
SCC3193	wild type	Pirhonen *et al*. (1988)
SCC3060	*expA*::Km^R	Pirhonen *et al*. (1991)
SCC3009	*expS*::Km^R	Pirhonen *et al*. (1991)
SCC3006	*aepA*::Km^R	Pirhonen *et al*. (1991)
SCC1935	*pehR*::Km^R	Saarilahti *et al*. (1992)
SCC500	*expA*::Km^R, *kdgR*::miniTn5Cm^R::*gusA*	I
SCC510	*kdgR*::miniTn5Cm^R::*gusA*	I
SCC519	*pmrB*::*cat*, Cm^R	II
SCC521	Δ*pmrA*::*cat*, Cm^R	II
SCC525	Δ*ryhB*::*cat*, Cm^R	III

Table 6. Plasmids used in studies I-III.

Plasmid	Relevant property	Reference/source
pGEM-T	Ap^R, cloning vector	Promega
pBluescript SK+	Ap^R, cloning vector	Stratagene
pPRG	Ap^R Cm^R, miniTn^5Cm^R::*gusA* in pUT	Marits *et al.* (1999)
pRA903	*expA* in pBluescript	Eriksson *et al*. (1998)
pHJH23	*kdgR* in pBluescript	I
pDR540-127	*pmrB* cytoplasmic domain in pDR540	II
pHJH44	1.5-kb *Eco*RI fragment from *pmrB* positive λ DASH II clone in pBluescript	II
pHJH45	6.5-kb *Eco*RI fragment from *pmrB* positive λ DASH II clone in pBluescript	II
pHJH46	*pmrAB*, as a 2.048 kb fragment in pGEM-T	II
pHJH46-1	*pmrAB*::*cat* in pGEM-T	II
pHJH48	2.923-kb fragment containing *pmrAB* in pGEM-T	II
pTPE114	Δ*pmrA*::*cat* in pGEM-T	II
pHJH49	1.31-kb *Hind*IIII fragment from pHJH45 containing *pmrA* and 3' end of *pmrC* in pBluescript	II
pHJH50	3.6-kb *Hind*IIII fragment from pHJH45 containing 5' part of *pmrC* in pBluescript	II
pHJH51	*pmrB*, as a 1091-bp fragment PCR-amplified from SCC3193 genomic DNA, cloned in pBluescript SK	II
pHJH52	*pmrA*, as a 665-bp fragment PCR-amplified from SCC3193 genomic DNA, cloned in pBluescript SK	II
pGP704	Ap^R, suicide vector	Miller and Mekalanos (1988)
pMUT61	6.1-kb *Sau*3A fragment containing *celV1* in pMUT201	Mäe *et al*. (1995)
pAMV1	6.1-kb *Sal*I fragment from pMUT61 containing *celVI* cloned into *Sal*I site in pBluescript	Mäe *et al*. (1995)
pHJH54	273-bp PCR fragment containing *ryhB* cloned into *EcoR*V site in pBluescript	III
pHJH57	1.175-kb PCR fragment containing *sapA* and upstream region of *ryhB* in pBluescript	III
pHJH58	0.93-kb *cat* in pHJH57	III
pHJH62	1.692-kb PCR fragment containing *celV1* in pHJH58	III
pHJH63	3.8-kb fragment containing Δ*ryhB::cat* in pGP704	III

Table 7. Methods used in studies I-III.

Method	Described and used in
Arbitrary PCR	I
Chemiluminescent detection	I, II, III
DNA techniques	I, II, III
DNA sequencing	I, II, III
Dot-blot analysis	II
Extracellular enzyme assays	I, II, III
Iron susceptibility assay	II
Marker exchange mutagenesis	II, III
Northern blot analysis	I, II, III
Phage library screening	II
Polymyxin resistance test	II
RNA isolation	I, II, III
RNA secondary structure prediction	III
Survival test *in planta*	II
T4GT7 transduction	I
Transposon mutagenesis	I
Virulence test	I, II, III

D. RESULTS AND DISCUSSION

1. ExpA response regulator and KdgR repressor mediate their function through the RsmA-*rsmB* RNA system

1.1. Inactivation of *kdgR* partially suppresses the avirulent phenotype of an *expA* mutant

To elucidate the control of the ExpA response regulator and its putative interactions with other regulators, we searched for second-site mutants restoring the wild-type phenotype to the extracellular enzyme-negative and avirulent *expA* mutant strain. Out of approximately 10 000 colonies screened, one mutant showed a clear restoration of protease production. The insertion was shown to be located in the *kdgR* gene. The *kdgR* gene encodes a protein, IclR-type repressor, known to negatively control extracellular enzyme genes in Ecc_{Ecc71} (Liu *et al*., 1999). $\text{KdgR}_{\text{SCC3193}}$ showed 97% amino acid identity to $\text{KdgR}_{\text{SCRI193}}$ and $\text{KdgR}_{\text{Ecc71}}$, suggesting a similar role of this repressor in Ecc_{SCC3193}.

The mutated *kdgR* allele was transferred to the wild-type background to verify its role in $Ecc_{\text{SCC3193.}}$ The production of extracellular enzymes cellulase, polygalacturonase, pectate lyases and protease was explored in *expA*, *expA kdgR*, *kdgR* and wild-type backgrounds (I, Figure 1B and C). The levels of extracellular enzymes were clearly derepressed in the *expA kdgR* double mutant in comparison with the *expA* mutant strain. However, the production of these enzymes did not reach the levels observed in the wild-type strain, underlining the importance of ExpA in their activation. Interestingly, the $kdgR_{SCC3193}$ mutant showed increased production of pectate lyases and protease, in accordance with the results from Ecc_{Ecc71} (Liu *et al*., 1999), but the production of cellulase and polygalacturonase did not increase significantly from the wild-type level. Furthermore, inactivation of *kdgR* in the *expA* mutant partially restored the virulence on potato leaves (I, Figure 2). Disease symptoms caused by the $kdgR_{SCC3193}$ mutant were similar to those of the wild-type strain. This is in contrast to the results from Ecc_{Ecc71}, where inactivation of *kdgR* clearly caused more extensive maceration of celery petioles than in the wild type (Liu *et al*., 1999). Differences in virulence of the *kdgR* mutants between Ecc_{SCC3193} and Ecc_{Ecc71} could be due to the different host plants used.

The expression of *celV1*, *pehA* and *prtW* was determined by Northern analyses to verify that the differences seen in the level of extracellular enzymes produced were caused by changes at the transcriptional level. The levels of *celV1*, *pehA* and *prtW* transcripts were clearly increased in the *kdgR expA* mutant strain, although not to the wild-type level (I, Figure 3A). Expression of *celV1*, *pehA* and *prtW* was also slightly elevated in the *kdgR* mutant as compared with the wild-type strain. Liu *et al*. (1999) showed that expression of the *pel-1*, *peh-1*, *hrpN* and *celV* genes were two- to fivefold higher in the $kdgR_{Ecc71}$ mutant than that in the wild type. Introduction of the *expA* wild-type allele *in trans* into all mutants and into the wild-type background increased the expression of the extracellular enzyme gene studied (I, Figure 3B; Eriksson *et al*., 1998). In addition, overexpression of $kdgR_{\text{SCC3193}}$ decreased expression of the *celV1*, *pehA* and *prtW* genes, although repression by KdgR on these genes was incomplete. In Ecc_{Ecc71}, a similar reduction of the levels of *peh-1* and *hrpN* transcripts was observed when the $kdgR_{Ecc71}$ gene was introduced into the *kdgR* mutant (Liu *et al*., 1999).

However, expression of *pel-1* and *celV* was totally abolished in that analysis (Liu *et al*., 1999). In conclusion, these results show that both ExpA and KdgR regulators control a set of the same target genes encoding extracellular enzymes in *Ecc*.

1.2. Expression of *rsmA* and *rsmB* RNA is controlled by ExpA and KdgR

Liu *et al*. (1999) showed by gel mobility shift assay that $KdgR_{Ecc71}$ bound directly on the promoter regions of the *pel-1* and *peh-1* genes and further within the transcriptional unit of the *rsmB* gene, suggesting that KdgR was preventing the elongation of transcription. In our study, suppression of the *expA* mutant phenotype by inactivation of the *kdgR* raised the question of whether the ExpA would also have an effect on the level of the *rsmB* RNA regulator. Moreover, as the *rsmB* RNA was reported to neutralize the effect of RsmA, a RNA-binding protein promoting message decay (Liu *et al*., 1998), we also included the *rsmA* gene in our studies. Our results showed that expression of *rsmB* was clearly decreased in the *expA* mutant (I, Figure 3A). This finding is in agreement with that observed in another *Erwinia* strain, Ecc_{Ecc71} (Cui *et al*., 2001). Interestingly, while expression of *rsmA* encoding a global repressor of virulence was increased in the $expA_{SCC3193}$ mutant (I, Figure 3A), this effect was not seen in Ecc_{Ecc71} (Cui *et al*., 2001). In addition, although expression of *rsmB* was increased in the *expA kdgR* mutant to the wild-type level, the expression of extracellular enzyme genes was not. This result suggests that control of the extracellular enzymes by ExpA was partially exerted through the RsmA-*rsmB* RNA, yet this control likely involves additional mechanism(s).

How does ExpA regulate *rsmB* RNA? Although the ExpA contains a DNA-binding domain and its orthologues affect expression of a number of different genes, in only one report has *Salmonella* SirA (ExpA orthologue) been shown by gel mobility shift assay to bind to the promoter regions of the *csrB* and *hilA* genes (Teplitski *et al*., 2003). Phosphorylation of SirA was not obligatory for binding to the *csrB* and *hilA* promoters (Teplitski *et al*., 2003). In our studies, overexpressed $ExpA_{SCC3193}$ protein did not bind to the *rsmB* in gel mobility shift assays, suggesting that the effect of ExpA on *rsmB* RNA could be indirect in $Ecc_{SCC3193}$. However, the overall nucleotide identity between the *csrB* of *Salmonella* and the *rsmB* of $Ecc_{SCC3193}$ was relative low. Furthermore, a close homologue of the *hilA* gene encoding a *Salmonella* invasion regulator has not been found in *Eca* or in other enterobacterial genomes. Therefore, binding of ExpA to its target genes may have evolved differently in enterobacterial species.

We demonstrated a novel positive control of the global repressor *rsmA* by $KdgR_{SCC3193}$. Expression of *rsmA* was decreased in the $kdgR_{SCC3193}$ mutant, and overexpression of *kdgR* in the *kdgR* mutant background clearly restored expression of the *rsmA* to the wild-type level (I, Figure 3A and B). This is contrary to the results with Ecc_{Ecc71}, where $KdgR_{Ecc71}$ had an effect on *rsmB* transcription but not on RsmA production. However, authors have suggested dual action of HexA and RsmC repressors on expression of both *rsmA* and *rsmB* genes (Cui *et al*., 1999; Liu *et al*., 1999; Mukherjee *et al.,* 2000; Chatterjee *et al*., 2002b). It is noteworthy that in Ecc_{Ecc71} the regulatory factors GacA-GacS, RsmC-RsmA-*rsmB* RNA, HexA and KdgR were suggested to control expression of the *hrp* regulon and specific Hrp regulators encoded by it, which in turn control expression of *hrpN* (Chatterjee *et al*., 2002b). In contrast, $Ecc_{SCC3193}$ was shown to be a *hrpN*-deficient strain (Mattinen *et al*., 2004). Thus, these

differences in virulence regulation could be due to a different set of virulence genes expressed by these two *Ecc* strains. The lack of Harpin molecule in $Ecc_{SCC3193}$ might have had some influence on how these regulatory pathways evolved.

1.3 Putative interaction with other known regulatory factors

A transposon mutagenesis approach to screen suppressors of the *expA* was also applied to the *expS* mutant (Pirhonen *et al*., 1991), which is defective in a putative sensor kinase partner of ExpA response regulator (Eriksson *et al*., 1998). This mutagenesis resulted in identification of the *rsmC* gene that encodes a regulator of the RsmA-*rsmB* RNA system (Cui *et al*., 1999; Shih *et al*., 1999; data not shown). Inactivation of the *rsmC* suppressed the *expS* phenotype and led to increased extracellular enzyme production and virulence *in planta* (data not shown). The $rsmC_{SCC3193}$ mutant showed decreased growth and the *rsmC* gene contained 5'UTR expressed only during the earlier stages of logarithmic growth, as described for Ecc_{MH1000} but not for Ecc_{Ecc71} (Shih *et al*., 1999; Cui *et al*., 1999; data not shown). Furthermore, screening for second-site suppressors of an *aepA* mutant (SCC3006, Pirhonen *et al*., 1991) showing decreased production of extracellular enzymes also resulted in identification of the inactivated *rsmC* gene (data not shown). These results imply that the ExpS-ExpA TCS together with the AepA activator protein might interact and exert their regulatory function through the RsmC-RsmA-*rsmB* system in $Ecc_{SCC3193}$.

2. PmrA-PmrB two-component system controls virulence

2.1. Sensor kinase PmrB, a suppressor of *expA* mutation

We were particularly interested in determining whether any downstream regulatory factor exist in the regulatory hierarchy of ExpA. In addition to the second-site transposon mutagenesis, another approach was used to answer this question. A plasmid library containing genomic fragments of $Ecc_{SCC3193}$ was introduced into the *expA* mutant, and the clones suppressing the cellulase-negative phenotype of the *expA* mutant were selected for further studies. A similar approach has been successfully applied to obtain suppressors of response regulator mutants in *E. coli* (Nagasawa *et al*., 1992, 1993). The most interesting clone restoring the production of cellulase in the *expA* mutant background showed sequence similarity to the cytoplasmic part of sensor kinase proteins. The corresponding gene with its flanking regions was isolated, and an operon designated by *Salmonella pmrCAB* was identified (II, Figure 1A; Roland *et al*., 1993). The two first open reading frames (ORF) consisted of a putative cytoplasmic membrane-bound protein and a response regulator that showed 59% amino acid identity to the PmrC and PmrA (II, Figure 1B) of *Salmonella enterica* serovar Typhimurium, respectively. The last ORF encoded the sensor kinase protein PmrB, which showed 57% identity to the *Salmonella* PmrB (II, Figure 1C). The *pmrCAB* operon was also found to contain a putative conserved PmrA binding site (II, Figure 1A; Wösten and Groisman, 1999; Aguirre *et al*., 2000).

2.2. Role of PmrA-PmrB in virulence

The TCS PmrA-PmrB had been characterized from another enterobacterial species, the animal pathogen *Salmonella enterica* serovar Typhimurium, which exhibits an intracellular infective life cycle (Roland *et al*., 1993; Gunn and Miller, 1996; Soncini and Groisman, 1996). Furthermore, another TCS, PhoP-PhoQ, is known to control the PmrA-PmrB via a small PmrD regulatory protein in *Salmonella* (Roland *et al*., 1994; Gunn and Miller, 1996; Kox *et al*., 2000; Kato *et al*., 2003). The PhoP-PhoQ has been suggested to be the most important virulence regulatory system in *Salmonella*, controlling more than 40 target genes (Groisman, 2001), whereas in *Ecc* the orthologous TCS PehR-PehS was found to activate only one of the extracellular enzyme genes, *pehA*. Hence, parts of the regulatory network pathways may have been evolved divergently in these enterobacterial pathogens, especially as the presence of a highly homologous *pmrD* gene product connecting these two TCSs in *Erwinia* seems to be unlikely.

To explore the role of TCS PmrA-PmrB in virulence of *Ecc*, we constructed *pmrA* and *pmrB* mutants. The *pmrB* mutant showed decreased maceration capacity in potato tubers as well as in Arabidopsis leaves, and its survival was reduced *in planta* (II, Figure 2A, B and C). Interestingly, inactivation of *pmrA* caused no significant reduction in virulence, although it did reduce bacterial survival in Arabidopsis. The reduced virulence of the *pmrB* mutant was at least partially dependent on the decreased production of extracellular enzymes and down-regulation of corresponding genes *prtW*, *celV1*, *pehA* and *pelB* (II, Figure 3A and Table 1). In contrast, the levels of these gene products in the *pmrA* mutant were somewhat higher, suggesting that PmrA might have a negative effect on the expression of extracellular enzyme genes. However, when the *pmrB* mutant was complemented by introducing the $pmrB^+$ wild-type allele, overexpression of the *pmrA* gene in wild-type or in mutant backgrounds had no significant effect on extracellular enzyme levels. These apparently contradictory results could be explained in part by the observation that the expression of *pmrA* was up-regulated in the *pmrB* background (II, Figure 4). Alternatively, the PmrB could cross-react with yet another unknown regulatory factor.

In *Salmonella*, initial studies suggested that PmrA might have a role in adaptation to neutrophils during pathogenesis (Roland *et al.*, 1993), but PmrA has subsequently been shown to have no impact on macrophage survival and growth within host tissues, instead being important during the early stages of natural infection through the oral route (Gunn *et al*., 2000). However, the *Salmonella pmrB* gene was demonstrated to be required for survival in neutrophils (Heithoff *et al*., 1997). Consequently, the *pmrB* genes of both *Ecc* and *Salmonella* seem to affect virulence more seriously than their cognate response regulator *pmrA*, suggesting importance of sensor histidine kinase in signal detection.

2.3. PmrA-PmrB modifies lipopolysaccharides

In *Salmonella*, the PmrA-PmrB TCS controls genes that modify LPS structures in a manner depending on both pH and Mg^{2+}concentration (Groisman *et al*., 1997; Gunn *et al*., 1998). The PmrA-PmrB TCS is needed to activate expression of the *pmrHFIJKLM* operon essential for LPS modifications leading to a reduction in the net negative charge of lipid A,

which in turn provides resistance against cationic antimicrobial peptide polymyxin B (PM) (Groisman *et al*., 1997; Gunn *et al*., 1998, 2000).

To investigate whether *Ecc* PmrA-PmrB TCS also participates in similar modifications of LPS, we tested polymyxin resistance of the *pmrA* and *pmrB* mutants as well as of the wild-type strain. The *pmrA* mutant showed an increased sensitivity against polymyxin B (II, Figure 5), which is in accordance with the results observed in *Salmonella* (Groisman *et al*., 1997). The *Ecc pmrB* mutant, by contrast, revealed significantly increased resistance against PM and complementation of the *pmrB* mutant was shown to restore the PM sensitivity to the wild-type level (II, Figure 5A and B). This is unlike in *Salmonella*, where the *pmrB* mutant was reported to exhibit a PM-sensitive phenotype similar to the *pmrA* mutant (Kox *et al*., 2000; Wösten *et al*., 2000). It is worth noting, however, that the studies with the *Salmonella pmrA505* mutant containing a single amino acid substitution resulted in a high-level PM resistance supposed to be due to constitutive expression of the *pmrA* gene (Gunn *et al*., 1998). In *Erwinia*, inactivation of *pmrB* caused overexpression of the *pmrA* (II, Figure 4A), which could explain the increased PM resistance of the *pmrB* mutant. Interestingly, it was recently shown that inactivation of *pmrA* in *Yersinia pseudotuberculosis* did not affect PM resistance, and the authors suggested that the target genes of the PmrA-PmrB TCS differ substantially in distinct bacterial species (Marceau *et al*., 2004). However, in *Pseudomonas aeruginosa*, the PmrA-PmrB TCS affected PM resistance (McPhee *et al*., 2003; Moskowitz *et al*., 2004), although the similarity of *P. aeruginosa* PmrA and PmrB proteins was significantly lower than that of *Y. pseudotuberculosis* and *Ecc* PmrA and PmrB (Marceau *et al*., 2004).

Since the PmrA-PmrB TCS affected PM resistance in *Ecc*, one might ask if there is any relevance of this system in plant infection? The *pmrB* mutant defective in production of extracellular enzymes could have elevated resistance against the cationic antimicrobial peptides produced by a host plant. The increased sensitivity of the *pmrA* mutant against such peptides could be compensated by overproducing of the extracellular enzymes, leading to plant tissue maceration. Therefore, one might speculate that inactivation of one component of the PmrA-PmrB TCS does not necessarily lead to a deleterious phenotype, but instead the bacteria could overcome such a deficiency by increasing certain survival (in *pmrB*) or virulence (in *pmrA*)-favouring characteristics.

2.4. Response of PmrA-PmrB to pH and Mg^{2+}

In *Salmonella*, as well as in other Gram-negative bacterial species, Mg^{2+} limitation and mild acidic pH have been found to increase PM resistance (García Véscovi *et al*, 1996; Groisman *et al*., 1997; Macfarlane *et al*., 1999; Brodsky *et al*., 2002). Similar to *Salmonella*, the PM resistance was clearly increased in *Ecc* when the cells were grown in a medium at pH 5.8 that contained low concentration of Mg^{2+} (II, Figure 6). However, when the *pmrB* mutant was grown in the same minimal medium at neutral pH and a low concentration of Mg^{2+}, the cells were sensitive to PM. In *Salmonella*, by contrast, neutral pH and low Mg^{2+} concentration favoured PM resistance, whereas PM resistance decreased in neutral pH conditions when Mg^{2+} ions were present at a millimolar concentration (Groisman *et al*., 1997).

The biological relevance of such an increase in PM resistance at mild acidic pH and a low level of Mg^{2+} could be explained by considering the infection process of *Ecc in planta*. During early infection this intercellular pathogen encounters the apoplastic fluid of plant with

a pH around 6 and a low concentration of Mg^{2+} and other cations. Resistance against antimicrobial peptides produced by the host plant might be necessary at this stage. The low concentration of Mg^{2+}/Ca^{2+} activates production of polygalacturonase, which causes the initial damages to the plant cell wall. This then leads to ion leakage and an increase in Mg^{2+}/Ca^{2+} concentration and pH. Conceivably, *Ecc* no longer needs to exhibit resistance to antimicrobial peptides under conditions in which the infection is already in progress and a number of other extracellular enzymes have been employed to destroy the plant tissue.

2.5. PmrA is necessary to avoid iron toxicity

The *Salmonella* histidine kinase PmrB contained two putative iron-binding motifs (ExxE) located in the periplasmic region (Wösten *et al*., 2000) and these motifs were also found in the *Ecc* PmrB (II, Figure 1C). The *Salmonella* PmrA-PmrB was shown to be activated by extracytoplasmic iron and to provide protection against oxygen-independent toxicity of iron (Wösten *et al*., 2000). Recently, it has been suggested that in the absence of a specific iron signal the phosphorylated PmrA is actually dephosphorylated by the PmrB sensor kinase in *Salmonella* (Kato and Groisman, 2004). The *Salmonella pmrA* mutant proved to be sensitive to $FeSO_4$ when the cells were grown in a mildly acidic liquid minimal medium containing a low concentration of Mg^{2+} (Wösten *et al*., 2000). However, in *Erwinia*, we did not observe any significant difference in these same conditions between the *pmrA* mutant, the *pmrB* mutant and the wild type (II, Figure 7B). However, the *Erwinia pmrA* mutant was sensitive to 500 μM $FeSO_4$ at mildly acidic conditions and high levels of Mg^{2+} (II, Figure 7A). Furthermore, the *Erwinia* strains survived an excess of iron when the pH was neutral in the growth medium (II, Figure 7A). Therefore, the pH seems to be a more important factor than Mg^{2+} concentration in PmrA-mediated tolerance to high extracytoplasmic iron concentration in *Erwinia*. This might reflect the situation in the apoplastic fluid, with damages to the plant cell wall leading to an increase in ion concentration, including an increase in iron.

Recently, *E. coli* K12 grown on solid medium under mild acid conditions was shown to require the BasR-BasS TCS (PmrA-PmrB orthologues) to survive high concentrations of iron (Nagasawa *et al*., 1993; Hagiwara *et al*., 2004). On a solid medium, 0.1 mM $FeSO_4$ restricted the growth of *Salmonella pmrA* and *pmrB* mutants, while toxic iron concentrations for *Erwinia pmrA* and *pmrB* and for *E. coli basSR* mutants were 1 mM and 1.5 mM, respectively (II, Figure 7C; Wösten *et al*., 2000; Hagiwara *et al*., 2004). The results show that *Erwinia*'s capacity to survive excess iron at low pH on a solid medium is significantly higher than in a liquid medium. This might indicate that the *Erwinia* PmrA-PmrB TCS is also necessary for bacterial survival in soil, as suggested for *Salmonella* (Chamnongpol *et al*., 2002).

3. Novel RyhB-like RNA controls expression of the *celV1* gene

3.1. Characterization of a small, iron-responsive RyhB RNA

An unidentified approximately 100-nt signal was detected in Northern analysis with a *celV1*-specific probe covering the upstream region of the *celV1* promoter. This unknown

RNA was induced strongly in cells grown in minimal medium, but it was also present in cells grown in L-medium at the early logarithmic phase (III, Figure 1A). The published *celV1* gene sequence contained 267 bp upstream from its translational start codon, and sequence comparison at the nucleotide level resulted in a high degree of homology to a small 90-nt *ryhB* (*sraI*) RNA of *E. coli* (Argaman *et al*. 2001; Wassarman *et al*. 2001; III, Figure 1C). Comparison of the $ryhB_{SCC3193}$ with the genome sequence of $Eca_{SCRI1043}$ (Bell *et al*., 2004) and with the *celV* gene of $Ecc_{SCRI193}$ (Cooper and Salmond, 1993) revealed a high identity between these sequences located upstream of the *celV* gene in these *Erwinia carotovora* strains (III, Figure 1B and C).

Small, non-coding RNAs have been identified in several genome-wide searches in *E. coli* (Gottesman, 2002; 2004; Hershberg *et al*., 2003; Vogel *et al*., 2003). However, data collected by computational means or even detected by microarray are considered inadequate. One of the studied small RNAs, known to be expressed and functional, is the *E. coli* RyhB RNA and its functional analogues, PrrF RNAs, in *Pseudomonas aeruginosa* (Massé and Gottesman, 2002; Wilderman *et al*., 2004). In *E. coli*, the RyhB RNA was verified to regulate genes involved in iron acquisition and storage and to be repressed by Fur in high iron concentrations. In addition, the RyhB RNA bound to its target RNAs, triggering their degradation in a process dependent on RNA chaperone Hfq and endoribonuclease RNaseE (Massé *et al*., 2003). The putative Fur-binding sites were also present in the promoter region of $ryhB_{Ecc}$ (III, Figure S1), and expression of the $ryhB_{Ecc}$ was highly induced when free iron was depleted from bacterial cells (III, Figure 3C). This suggests that $RyhB_{Ecc}$ could be responsible for controlling intracellular iron homeostasis, as it does in *E. coli*.

3.2. RyhB RNA function has evolved differently in *Ecc*

A deletion mutant of the $ryhB_{Ecc}$ gene was constructed to elucidate its function in *Ecc*. Functional analysis of the $RyhB_{Ecc}$ revealed several differences as compared with the $RyhB_{E.coli}$. Firstly, expression of $ryhB_{Ecc}$ was induced at an early logarithmic growth phase in L-medium (III, Figure 3B), whereas the $RyhB_{E.coli}$ was shown to be expressed when the cells reached the end of logarithmic growth in same medium (Argaman *et al*., 2001). Secondly, overexpression of the $ryhB_{Ecc}$ in the wild-type background did not restrict the growth of *Ecc* on succinate (III, Table 1), unlike in *E. coli* (Massé and Gottesman, 2002), indicating that their functions might differ. Thirdly, inactivation of $ryhB_{Ecc}$ had no effect on expression of the *sdhCDAB* operon encoding a succinate dehydrogenase enzyme, which requires Fe^{2+} ions for activity (III, Figure 4). In contrast, the $RyhB_{E.coli}$ was suggested to repress expression of the *sdhCDAB* under iron-limiting conditions (Massé and Gottesman, 2002).

The secondary structure prediction of the $RyhB_{Ecc}$ resulted in a different and more complex structure compared with the $RyhB_{E.coli}$, especially the bases important for interaction with the *sodB* target gene and $RyhB_{E.coli}$ (Večerek *et al*., 2003) were embedded in stacked structures in $RyhB_{Ecc}$ (III, Figure 2). The $sodB_{E.coli}$ encoding an iron superoxide dismutase was shown to be another direct target of $RyhB_{E.coli}$ (Večerek *et al*., 2003; Geissmann and Touati, 2004). However, the *sodB* gene is most likely absent from *Erwinia* genomes (Santos *et al*., 2001), indicating that in *Ecc* the resistance mechanism against active oxygen species likely includes other components than in *E. coli*.

Interestingly, inactivation of the *ryhB*$_{Ecc}$ resulted in earlier induction and increased expression of the *celV1* gene encoding the major cellulase in *Ecc*$_{SCC3193}$ (III, Figure 5A and B), suggesting that it might function as a repressor of the *celV1*. A region of complementarity was found between the *celV1* and the RyhB$_{Ecc}$ RNA, possibly suggesting a direct interaction (III, Figure 5C). However, the possibility that the RyhB$_{Ecc}$-mediated effect on the *celV1* gene is indirect can not be ruled out. Furthermore, comparison of RyhB$_{Ecc}$ and RyhB1$_{Eca}$ with the genome sequence of *Eca* (Bell *et al*., 2004) revealed another putative target gene for RyhB RNA, *dsbC* encoding periplasmic disulfide oxidoreductase. Intriguingly, transcription of the *celV* was up-regulated in the *Ecc dsbA* mutant encoding a major protein in the periplasmic Dsb system (Vincent-Sealy *et al*., 1999). This could imply that the target genes of *Ecc* RyhB have differentially evolved from those reported for *E. coli* RyhB RNA.

3.3. Iron limitation increases expression of extracellular enzyme genes

The identification of iron-responsive RyhB RNA led us to explore the effects of iron limitation on the expression of extracellular enzyme genes. Iron limitation has been shown to induce expression of pectate lyase-encoding genes of *Echr* (Expert, 1999; Franza *et al.*, 1999, 2002). In *Ecc,* iron limitation studies have focused on siderophores involved in iron acquisition, but these were reported to be unimportant for pathogenesis (Expert, 1999).

Iron-limiting conditions were achieved by adding an iron chelator to bacterial cells. This mimics the plant environment at the initial stages of infection, when the availability of iron is restricted (Expert, 1999). A depletion of free iron from the *Ecc* wild type caused a clear and rapid increase in expression of cellulase-, polygalacturonase- and protease-encoding genes but not of pectate lyase *pelB* (III, Figure 5A). Interestingly, when the *pmrA* mutant (II) cells were subjected to the iron-chelating agent, the negative regulation of the extracellular enzyme genes, including the *pelB*, was enhanced compared with the wild type (III, Figure 5A; II, Figure 3A). This supports the idea that the signal activating the PmrA-PmrB TCS is indeed iron and that it is required to release the negative control of PmrA on the extracellular enzyme genes (III, Figure 6). In conclusion, in *Ecc*, as in other pathogenic bacteria, iron limitation is one of the important signals to trigger virulence gene expression.

3.4. ExpA controls succinate utilization and expression of the *sdhCDAB* operon

The identification of a novel, small regulatory RNA in *Ecc* led us to investigate the ability of other regulatory *Ecc* mutants to utilize the carbon sources succinate and fumarate. Interestingly, the *expA* mutant was unable to utilize succinate or fumarate as the primary carbon source (III, Table 1). Furthermore, as the *expA* mutant of *Ecc* showed a similar defect in utilizing succinate as described for the *fur* mutant of *E. coli* (Hantke, 1987; Massé and Gottesman, 2002), the possibility of an interaction between Fur and ExpA in the same regulatory pathway was investigated.

However, expression of neither *ryhB* nor *fur* was under the control of ExpA in *Ecc* (III, Figure 4). Strikingly, the expression level of *sdhCDAB* was increased in the *expA* mutant. Thus, the regulatory effect on the *sdhCDAB* was the opposite of that reported for the *fur*$_{E.coli}$ mutant. An explanation for the defect in the *expA* mutant in succinate utilization might be

inability to transport succinate into cells and putative ExpA-mediated control of the *dctA* gene encoding a C_4-dicarboxylate transporter. This is supported by transcriptome analysis, including all *E. coli* TCSs, where expression of the *dctA* was down-regulated in the *uvrY* (*expA* orthologue) mutant (Oshima *et al*., 2002). Interestingly, the *gacA* (*expA* orthologue) mutant of *Vibrio fischeri* was reported to be unable to utilize fumarate as a primary carbon source (Whistler and Ruby, 2003). Increased expression of the *sdhCDAB* in the *expA* mutant could be due to an increase in expression of the *rsmA* (I, Figure 3A and B). In *E. coli*, the RsmA homologue CsrA positively controls glycolysis, although it does not have any effect on the levels of key enzymes involved in the TCA cycle (Sabnis *et al*., 1995; Yang *et al*., 1996; Romeo, 1998; Wei *et al*., 2000; Pernestig *et al*., 2003).

The C_4-dicarboxylic acids succinate, fumarate and malate have been described to act as chemoattractants in plant-microbe interactions (Brencic and Winans, 2005). Strikingly, also the *expS* and *aepA* mutants were unable to utilize succinate and fumarate (data not shown). Since the *expS* likely encodes the cognate histidine kinase for *expA*, and *aepA* was induced by plant extracts (Liu *et al*., 1993), it would be tempting to speculate that the C_4-dicarboxylic acids are plant-derived signals detected by these regulators.

E. CONCLUDING REMARKS

The regulatory network controlling virulence in the plant pathogen *Erwinia carotovora* ssp. *carotovora* is complex. A part of this network was revealed here by studying the response regulator ExpA, a partner in a two-component system that has an important role in activation of virulence. The results indicate that ExpA controls the virulence determinants together with the KdgR repressor, and part of this effect is mediated through the RsmA-*rsmB* RNA system. Furthermore, identification of a TCS PmrA-PmrB and a small regulatory RyhB RNA suggests that the regulatory network, the complexity of which has been acknowledged for sometime, may still contain unknown regulatory components.

The identification of TCS PmrA-PmrB and its effect on virulence and resistance against antimicrobial peptides also in the plant pathogenic bacterium was an interesting result. Since both PmrA-PmrB and ExpS-ExpA TCSs affect expression of extracellular enzyme genes, and hence virulence, their putative interaction is intriguing, especially as the different TCSs are suggested to form a signal transduction network. However, this potential interaction requires further clarification. Control of the PmrA-PmrB on virulence gene expression is more likely indirect, but as the PmrA binding site is well conserved, the recently uncovered genome sequence of *Eca* provides an excellent tool to search for putative target genes of the *Ecc* PmrA response regulator.

Genome-wide studies have identified a number of new, small RNAs in the intergenic regions of *E. coli*. These small RNAs, which may have a regulatory function, are also additional members in regulatory cascades in bacteria. Identification of a small RyhB-like RNA in *Ecc* revealed that it was an iron-responsive RNA, although its function may have evolved differently. A similar difference has been suggested for the RsmA-*rsmB* RNA system, which targets a different set of genes than the CsrA-*csrB* RNA system. Therefore, the presumption that these small RNAs would function identically in different bacterial species might be misleading in regulatory cascade studies.

The RsmA repressor is one of the key components in different regulatory cascades of *Erwinia*. While the RsmA exerts negative control on virulence gene expression, it is itself subject to the control of several regulatory factors. The two global activators of virulence, ExpA response regulator and ExpI, responsible for quorum sensing, are negative regulators of *rsmA*. Conversely, the negative regulators of virulence, i.e. KdgR, HexA, RsmC and RpoS sigma factor, have a positive effect on expression of *rsmA*. It is noteworthy that in the control of RsmA variation appears to exist in the *Ecc* strains, which might be related to differences in pathogenicity factors such as motility and existence of *hrp* genes. Interestingly, the subspecies boundaries in *Erwinia carotovora* seem to be quite variable since some of the regulatory factors, e.g. ExpA and RyhB RNA, of *Ecc* strain SCC3193 are more closely related to the counterparts of *Eca* than those of *Ecc* type strains.

The regulatory components unique to *Erwinia* strains, such as the RsmC repressor and the AepA activator, also play a significant role in virulence regulation. ExpA-mediated control on C_4-dicarboxylic acid utilization is fascinating, and further studies with ExpS-ExpA, AepA and RsmC-RsmA-*rsmB* RNA are needed to reveal putative interactions between them and whether C_4-dicarboxylic acids play any role in signalling in the ExpS-ExpA system.

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