Quorum Sensing in the Plant Pathogen *Erwinia carotovora* subsp. *carotovora*: The Role of *expR_{Ecc}*

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The production of the main virulence determinants of the plant pathogen *Erwinia carotovora* subsp. *carotovora*, the extracellular cell wall-degrading enzymes, is partly controlled by the diffusible signal molecule *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL). OHHL is synthesized by the product of the *expI/carI* gene. Linked to *expI* we found a gene encoding a putative transcriptional regulator of the LuxR-family. This gene, *expR_{Ecc}*, is transcribed convergently to the *expI* gene and the two open reading frames are partially overlapping. The *ExpR_{Ecc}* protein showed extensive amino acid sequence similarity to the repressor EsaR from *Pantoea stewartii* subsp. *stewartii* (formerly *Erwinia stewartii* subsp. *stewartii*) and to the *ExpR_{Ech}* protein of *Erwinia chrysanthemi*. Inactivation of the *E. carotovora* subsp. *carotovora* *expR_{Ecc}* gene caused no decrease in virulence or production of virulence determinants in vitro. In contrast, there was a slight increase in the maceration capacity of the mutant strain. The effects of *ExpR_{Ecc}* were probably mediated by changes in OHHL levels. Inactivation of *expR_{Ecc}* resulted in increased OHHL levels during early logarithmic growth. In addition, overexpression of *expR_{Ecc}* caused a clear decrease in the production of virulence determinants and part of this effect was likely to be caused by OHHL binding to *ExpR_{Ecc}*. *ExpR_{Ecc}* did not appear to exhibit transcriptional regulation of *expI*, but the effect on OHHL was apparently due to other mechanisms.

The soft-rot erwinias, e.g. *Erwinia carotovora* subsp. *carotovora*, are considered to be opportunistic plant pathogens that are able to survive in the soil as saprophytes. In conditions favorable to the pathogen they can cause severe damage on the host plants by macerating different plant organs and tissues with multiple extracellular enzymes (Barras et al. 1994; Collmer and Keen 1986; Pérombelon and Kelman 1980). Among these enzymes are cellulases (Cel), pectate lyases (Pel), polygalacturonase (Peh), and proteases (Prt). The production and secretion of these plant cell wall-degrading enzymes is of crucial importance for the virulence of *E. carotovora* subsp. *carotovora*, as demonstrated, e.g., by isolation of mutants that are affected either in enzyme production (*exp*, *aep*, *hor*, and *rex* mutants) (Andersson et al. 1999b; Eriksson et al. 1998; Jones et al. 1993; Murata et al. 1991; Pirhonen et al. 1991, 1993; Thomson et al. 1997, 1999) or secretion of these enzymes (*out* mutants) (Hinton and Salmond 1987; Murata et al. 1990; Pirhonen et al. 1991; Salmond and Reeves 1993).

Production of the extracellular, plant cell wall-degrading enzymes in *E. carotovora* subsp. *carotovora* is a tightly controlled process involving both positive (*ExpI*, *ExpA*, *ExpS*, *ExpM*, *Hor*, *AepA*, *RexZ*, *RsmB*) and negative (*RsmA*, *HexA*, *KdgR*, *RpoS*) global regulators (Andersson et al. 1999a, 1999b; Chatterjee et al. 1995; Cui et al. 1995; Eriksson et al. 1998; Harris et al. 1998; Jones et al. 1993; Liu et al. 1993, 1998, 1999; Mukherjee et al. 1996, 1998; Murata et al. 1991; Pirhonen et al. 1991, 1993; Thomson et al. 1997, 1999). We and others have earlier demonstrated that quorum sensing, a term coined by Fuqua et al. (1994), by accumulation of a diffusible signal molecule, *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL), is a central feature of this regulation in *E. carotovora* subsp. *carotovora* (Jones et al. 1993; Pirhonen et al. 1993). Expression of the virulence genes encoding plant cell wall-degrading enzymes is only activated when a sufficient amount of the signal molecule has accumulated. The synthesis of OHHL is directed by the *expI/carI* gene (Jones et al. 1993; Pirhonen et al. 1991, 1993; Swift et al. 1993) and avirulent *E. carotovora* subsp. *carotovora* *expI/carI* mutants have been shown to lack this diffusible signal molecule (Jones et al. 1993;

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McGowan et al. 1995; Pirhonen et al. 1993). Several related cellular communication systems based on diffusible chemical signals, mainly *N*-acyl-homoserine lactones (HSL), have been described (for reviews see Fuqua et al. 1994, 1996; Salmond et al. 1995). The paradigm of these systems is the regulation of bioluminescence in the marine bacterium *Photobacterium* (*Vibrio*) *fischeri* (Kaplan and Greenberg 1985). Expression of the structural genes (*luxCDABEG*) required for light production is dependent on *luxI*-directed synthesis of the same autoinducer as in *E. carotovora* subsp. *carotovora* (OHHL). Accumulation of this autoinducer in the growth medium results in complex formation with the *lux* gene-specific, DNA-binding protein LuxR. The LuxR-autoinducer complex activates expression of the *luxICDABEG* operon, creating a positive feedback loop that results in both enhanced production of autoinducer and light emission. Hence, *P. fischeri* is able to sense its own cell density and light is produced only when high enough cell density is reached (for review see Meighen 1991).

The LuxR protein of *P. fischeri* was the first autoinducer-responsive transcriptional activator described. It has been genetically dissected with point mutations and deletions (Choi and Greenberg 1991, 1992a, 1992b; Hanzelka and Greenberg 1995; Shadel et al. 1990; Slock et al. 1990). This analysis has suggested a modular structure for this regulator. The N-terminal part contains the autoinducer-binding domain (Hanzelka and Greenberg 1995) and the C-terminal half the DNA-binding domain (Choi and Greenberg 1992a). These two binding domains appear to be the most conserved areas in the LuxR family of proteins, which now consists of a large number of well-characterized transcriptional regulators (for review see Fuqua et al. 1994; Swift et al. 1996). Three LuxR homologues have been characterized in plant-pathogenic bacteria, CarR in *E. carotovora* subsp. *carotovora* (McGowan et al. 1995), EsaR in *Pantoea stewartii* subsp. *stewartii* (formerly named *Erwinia stewartii* subsp. *stewartii*) (von Bodman and Farrand 1995; von Bodman et al. 1998), and ExpR_{Ech} in *Erwinia chrysanthemi* (Nasser et al. 1998). CarR is an OHHL-dependent positive regulator of genes involved in the synthesis of the antibiotic carbapenem produced by certain *E. carotovora* subsp. *carotovora* strains. EsaR seems to act as a negative regulator of extracellular polysaccharide (EPS) production in *P. stewartii* subsp. *stewartii*, and *esaR* mutants constitutively produce this major virulence factor (von Bodman et al. 1998). The *E. chrysanthemi* expR_{Ech} mutant shows no clear phenotype, but it has been shown that the ExpR_{Ech} protein can interact with the regulatory regions of genes encoding Pels in vitro and also with the promoter of the *E. chrysanthemi* *expI* gene (Nasser et al. 1998; Reverchon et al. 1998).

The transcriptional regulator(s) that interacts with OHHL and regulates production of extracellular enzymes has not been identified in *E. carotovora* subsp. *carotovora*. In this article, we describe a gene, *expR_{Ecc}*, that is linked to *expI* and show that the *expR_{Ecc}* gene product is a structural analog of LuxR. Insertion mutants in *expR_{Ecc}* were not reduced in virulence and produced increased amounts of OHHL. Overexpression of *expR_{Ecc}* was shown to have a negative effect on the production of virulence factors and this seems to be partly due to ExpR_{Ecc} binding of OHHL.

RESULTS

The *expR_{Ecc}* gene is linked to *expI* and encodes a putative transcriptional regulator of the LuxR family.

Determination of the complete nucleotide sequence of the insert that complemented the *expI* mutation (Pirhonen et al. 1991, 1993) revealed the presence of another convergently transcribed open reading frame (ORF) downstream of *expI* (Fig. 1A). This gene, designated *expR_{Ecc}*, contained a 735-bp ORF preceded by a putative ribosome binding site (GAGG), 11 bp upstream from the postulated translation initiation codon. A putative translation termination codon (TAG) could also be identified. No sequences typical for either Rho-dependent or Rho-independent transcription termination could be detected. The *expI* and *expR_{Ecc}* ORFs were partially overlapping at their 3' ends (23 bp). The *expR_{Ecc}* ORF encodes a polypeptide of 245 amino acids with a calculated molecular mass of 28,540 Da and an estimated pI of 6.21. The size of the *expR_{Ecc}* gene product was confirmed by overproduction and selective labeling of the ExpR_{Ecc} polypeptide with the phage T7 expression system (Tabor 1990). Overexpression of the *expR_{Ecc}* gene resulted in the production of a single protein product with an apparent molecular mass of 27 kDa in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (data not shown), well in agreement with the calculated molecular mass. When protein data bases were searched with the deduced amino acid sequence of the ExpR_{Ecc} protein, it was found to belong to the LuxR family (Devine et al. 1989; Engebrecht and Silverman 1987). The ExpR_{Ecc} and LuxR polypeptides share identical amino acids throughout the whole sequence, showing an overall identity of 23%, with the C-terminal domain of the ExpR_{Ecc} polypeptide (181 to 245) being the most similar to that of the LuxR protein. This region of LuxR has been demonstrated to contain the DNA-binding domain (Choi and Greenberg 1991, 1992a; Slock et al. 1990). However, the highest degree of similarity between ExpR_{Ecc} and other proteins in the data bases was found to LuxR homologues from other plant pathogens, EsaR from *P. stewartii* subsp. *stewartii* and ExpR_{Ech} from *E. chrysanthemi* (Fig. 1B). EsaR has been shown to be involved in regulation of virulence in *P. stewartii* subsp. *stewartii* and acts as a repressor of virulence factors (von Bodman and Farrand 1995; von Bodman et al. 1998). ExpR_{Ech} from *E. chrysanthemi* is a regulatory protein that might be involved as one part of a complex regulatory system that controls quorum sensing (Nasser et al. 1998; Reverchon et al. 1998). ExpR_{Ecc} was shown to share 47% identity with EsaR and 61% identity with ExpR_{Ech} covering both the N-terminal, putative autoinducer-binding domain and the C-terminal, DNA-binding domain (Fig. 1B).

Phenotypic analysis of *expR_{Ecc}* mutants of *E. carotovora* subsp. *carotovora*.

Based on the results of initial complementation of the *expI* mutant (Pirhonen et al. 1993), we knew that *expR_{Ecc}* did not suppress the avirulent, enzyme-negative phenotype of *expI*. To characterize the role of ExpR_{Ecc} in the virulence of *E. carotovora* subsp. *carotovora*, two *expR_{Ecc}* mutants, SCC5002 and SCC5003, with selectable markers interrupting the *expR_{Ecc}* gene at *ClaI* and *SnaBI* sites, respectively, (Fig. 1A) were constructed. Both *expR_{Ecc}* mutants appeared to retain the ca-

capacity to produce autoinducer (OHHL) as well as extracellular plant cell wall-degrading enzymes (Cel, Peh, Pel, and Prt) and SCC5003 was used for further studies. To test whether an *expR_{Ecc}* mutation had any effect on virulence of *E. carotovora* subsp. *carotovora*, we performed a number of virulence tests. We first compared the virulence of SCC5003 with that of the wild type and found that they had similar virulence in the potato stem assay (data not shown). We then examined the viru-

capacity we characterized the production of extracellular enzymes during the different phases of bacterial growth in vitro. The results from these experiments showed rather similar levels of enzyme activity in SCC3193 and SCC5003. However, we repeatedly observed slightly higher levels of Pel activity,

indicating that lack of ExpR_{Ecc} might result in enhanced enzyme production (see Figure 2B). These results suggest that expR_{Ecc} may not encode a transcriptional activator of virulence genes in *E. carotovora* subsp. *carotovora*. In contrast, ExpR_{Ecc} might act as a negative regulator of genes encoding extracellular plant cell wall-degrading enzymes.

Multiple copies of expR_{Ecc} cause a reduction in the production of virulence factors.

To assess the possibility that ExpR_{Ecc} negatively regulates extracellular enzyme production we characterized the effect of expR_{Ecc} overexpression on Pel production. We used the multicopy plasmid pSA018 that carries the expR_{Ecc} gene under control of its own promoter and another plasmid, pRA801, that allows controlled overexpression of expR_{Ecc} upon induction by isopropyl β -D-thiogalactoside (IPTG). These plasmids were transferred separately into SCC3193 and SCC5003 and Pel activity was assayed from culture supernatants. When cells carrying control plasmids were compared, SCC5003 appeared to produce slightly more Pel. Introduction of pSA018 did not significantly affect growth but caused a 50% reduction in Pel activity (Fig. 2B), and pRA801 caused a similar reduction after induction by IPTG (data not shown). The accumulation of *pehA* and *pelB* transcripts was also markedly decreased in cells carrying pRA801 (data not shown). These results indicate that excessive amounts of ExpR_{Ecc} can cause significant reduction of extracellular enzyme production.

To test whether overexpression of expR_{Ecc} also affected maceration capacity on potato tubers we infected tubers with SCC3193 and SCC5003 carrying either pSA018 or a control plasmid. We found that expR_{Ecc} overexpression caused a significant decrease in maceration capacity (Fig. 2A). The above results suggested that ExpR_{Ecc} might act as a repressor of the extracellular enzyme encoding genes even though the effect of an expR_{Ecc} mutation was moderate. To test this hypothesis and to define whether the lack of ExpR_{Ecc} had any effect in the absence of OHHL, we constructed an expIR double mutant strain, SCC6005. We assayed OHHL production in SCC6005 and found that this mutant, as expected, failed to produce OHHL, similarly to the expI mutant strain SCC3065 (see below). We then compared the extracellular enzyme activities of the different strains as well as the accumulation of *pehA* and *pelC* transcripts from in vitro grown cultures. The double mutant SCC6005 exhibited reduced accumulation of transcripts (Fig. 3) and production of Peh and Pel (data not shown). In this respect the double mutant was not very different from the expI mutant strain (Pirhonen et al. 1993); however, it appeared to accumulate somewhat increased amounts of the *pehA* transcript. Assay of virulence on tobacco and potato showed that the double mutant SCC6005 was avirulent on both plant species, similarly to the expI mutant strain (data not shown). Taken together, these results suggest that ExpR_{Ecc} cannot be the main or the sole regulator that mediates the effect of OHHL.

An expR_{Ecc} mutant has increased levels of OHHL.

The increased production of extracellular enzymes in expR_{Ecc} mutants and the repression of enzyme gene expression by multicopy expR_{Ecc} could be explained by expR_{Ecc} controlling the level of OHHL. To characterize the effect of ExpR_{Ecc} on OHHL production we compared the levels of OHHL in culture supernatant of the expR_{Ecc} mutant SCC5003 and the

Fig. 2. Effect of ExpR_{Ecc} on tuber maceration and pectate lyase (Pel) production. **A**, An expR_{Ecc} mutant has increased maceration capacity, and expR_{Ecc} overexpression causes a significant reduction of maceration capacity. Assay was performed as described (Eriksson et al. 1998); each value is a mean of 16 inoculations with standard deviations. Results were analyzed statistically by means of the Student's *t* test. *P* value for the difference between SCC3193 and SCC5003 was 0.00119; between the same strains carrying the vector plasmid (pBluescript II), 0.176. *P* value for the difference between SCC3193 and SCC5003 carrying the vector plasmid or pSA018 (expR_{Ecc}) is 0.0107 and 0.000142, respectively. **B**, Suppression of Pel production by multiple expR_{Ecc} copies. SCC3193 (wild type, squares) and SCC5003 (expR_{Ecc} mutant, triangles) carrying a control plasmid, pBluescript II (closed symbols), or pSA018 (expR_{Ecc} open symbols). Samples were taken at time points indicated and Pel activity was measured from culture supernatants.

wild-type strain SCC3193. The result of this experiment shows that the *expR_{Ecc}* mutant produces more OHHL, especially during early exponential growth (2 to 4 h; OD₆₀₀ 0.2 to 0.7), while the level of OHHL is more similar between the two strains during late exponential growth and in stationary phase (Fig. 4A). These differences correspond to those seen in enzyme production and consequently the increased enzyme gene expression and maceration capacity of the *expR_{Ecc}* mutant strain could indeed be explained by this increase in OHHL. To determine the molecular mechanism of this control we tested whether ExpR_{Ecc} regulated *expI* gene expression transcriptionally. We first characterized the effect of ExpR_{Ecc} on transcription of *expI* with a transcriptional plasmid-borne *expI-uidA* fusion. This construct was introduced into the wild-type strain SCC3193 and into the *expR_{Ecc}* and *expI* mutant strains SCC5003 and SCC3065, respectively. Assay of β -glucuronidase (GUS) activity did not show any significant differences between these strains (Table 1). These results suggest that *expI* is not transcriptionally regulated by ExpR_{Ecc} and that the transcription of *expI* does not require OHHL. Northern (RNA) blot analysis was used to investigate whether the accumulation of *expI* transcript was affected by an *expR_{Ecc}* mutation or by overexpression of *expR_{Ecc}*. We did not find any significant differences in the level of *expI* transcript in an *expR_{Ecc}* mutant or in cells carrying pSA018 when compared with wild-type cells (data not shown). Taken together, our results indicate that ExpR_{Ecc} does not act as a direct repressor of *expI*. Instead, inactivation of *expR_{Ecc}* seems to cause increased levels of OHHL by other mechanism(s). One possibility is that ExpR_{Ecc} binds OHHL, thus decreasing the levels of free OHHL. According to this hypothesis, ExpR_{Ecc} could affect extracellular enzyme production indirectly by binding OHHL, thus decreasing OHHL availability to other regulators directly involved in OHHL-dependent activation/derepression of virulence genes. To investigate this further, we tested whether addition of synthetic OHHL could relieve the suppression of Pel production when *expR_{Ecc}* was overexpressed in the wild-type strain (cf. Figure 2). This experiment showed that the suppression caused by pSA018 was relieved stepwise by addition of 0.1, 1, and 5 μ g of OHHL per ml (Fig. 4B), suggesting that the suppression at least partly is caused by ExpR_{Ecc}-mediated binding of OHHL. The increase in Pel activity was up to 86% in the case of SCC3193 carrying pSA018, compared with a maximum of 29% in cells carrying a control plasmid. However, the Pel activity did not return to the levels in cells carrying a control plasmid at any OHHL concentration

tested. As a control in this experiment we used the *expI* mutant strain SCC3065 (Pirhonen et al. 1991, 1993) that, as expected, was complemented by the addition of OHHL.

DISCUSSION

The quorum sensing mechanism has been extensively studied in many bacterial species during recent years. This system has been found to regulate several different properties, e.g., light production, plasmid conjugation, and production of virulence factors and antibiotics (reviewed by Fuqua et al. 1994, 1996; Salmond et al. 1995). However, it seems that the function of the system and the organization of the genes involved are far more diverse and complex than first anticipated. In most cases the *luxI* homologue, which directs the synthesis of one or more homoserine lactone molecule(s), is linked to a *luxR* homologue that encodes a response regulator protein. However, there are examples of genes that are not linked, such as *traI/traR* in *Agrobacterium* spp. and *carI/carR*, which regulates antibiotic production in certain *E. carotovora* subsp. *carotovora* strains (Salmond et al. 1995). The DNA and auto-inducer-binding LuxR homologues have been found to act both positively and negatively, although most proteins appear to act as transcriptional activators. We have previously found a gene, designated *expR_{Ecc}*, linked to the *expI* gene, that directs the synthesis of OHHL (Pirhonen et al. 1993). In this study, we show that ExpR_{Ecc} is structurally similar to LuxR and that the *expI* and *expR_{Ecc}* ORFs are partially overlapping in their 3' ends and convergently transcribed. Although the similarity between the ExpR_{Ecc} and LuxR proteins was rather moderate (amino acid identity 23%, similarity 31%), other regulators of the same family exhibited a higher degree of amino acid identity with ExpR_{Ecc}. The most similar proteins were ExpR_{Ech} (61% amino acid identity) from *E. chrysanthemi* (Nasser et al. 1998), EsaR (47% amino acid identity) of *P. stewartii* subsp. *stewartii* (von Bodman and Farrand 1995), and CarR (34% amino acid identity) controlling carbapenem antibiotic production in certain strains of *E. carotovora* subsp. *carotovora* (McGowan et al. 1995). Thus, the regulators characterized in different erwinias (*P. stewartii* subsp. *stewartii* was previously named *Erwinia stewartii* subsp. *stewartii*) may form a distinct subfamily of LuxR-like proteins sharing a higher degree of amino acid identity with each other than with the other members of the family. The similar amino acids were spread throughout the whole polypeptide; however, regions with high similarity could be located both to the autoinducer-binding

Fig. 3. Phenotype of the *expIR* double mutant strain SCC6005. Accumulation of transcripts from *pehA* (Saarilahti et al. 1990) and *pelC* (Heikinheimo et al. 1995). Cells were grown in L medium with 0.4% polygalacturonic acid (PGA) and harvested at the time points indicated. SCC3193 is the wild-type strain and SCC3065 is an *expI* mutant strain (Pirhonen et al. 1991, 1993). Five micrograms of RNA was loaded in each lane.

region and, especially, to the C-terminal, DNA-binding domain (Choi and Greenberg 1991, 1992a, 1992b; Hanzelka and Greenberg 1995; Shadel et al. 1990; Slock et al. 1990). Consequently, the structural analysis of ExpR_{Ecc} indicates that it may be able to bind autoinducer and that it most likely is a DNA-binding protein.

The linkage between *expI* and *expR_{Ecc}* and the homology between ExpR_{Ecc} and other LuxR family members suggested

that it, after binding to OHHL, acted as a positive regulator of the virulence genes in *E. carotovora* subsp. *carotovora*. Therefore, we were surprised by the finding that the *expR_{Ecc}* mutants lacked a clear phenotype in our initial experiments. This suggests that the ExpR_{Ecc} protein may not be a transcriptional activator of the virulence genes; at least ExpR_{Ecc} is not essential for enzyme production and virulence in *E. carotovora* subsp. *carotovora*. However, the quorum sensing system might be highly redundant, meaning that other regulator(s) possibly involved cover the lack of ExpR_{Ecc} . Therefore, our data do not totally rule out the possibility that ExpR is a positive regulator under certain conditions.

Our results suggest that *expR_{Ecc}* may act negatively on extracellular enzyme production. The evidence for this negative regulatory role of *expR_{Ecc}* is as follows: (i) Inactivation of *expR_{Ecc}* causes increased levels of OHHL. (ii) Disruption of the *expR_{Ecc}* gene leads to a slightly increased Pel production in vitro as well as to somewhat increased macerating capacity of potato tubers. This is consistent with an enhanced production of cell wall-degrading enzymes in planta in the mutant strain, possibly due to the higher levels of OHHL. (iii) Introduction of additional copies of *expR_{Ecc}* in trans (pSA018) leads to a 50% reduction of Pel in the supernatant, presumably at least partly through binding of OHHL. (iv) Overexpression of *expR_{Ecc}* from a strong, inducible promoter (pRA801) resulted in complete or almost complete shut-off of virulence gene expression. This was observed both at the transcriptional level (with *pehA* and *pelB* target genes) and in the production of the corresponding plant cell wall-degrading enzymes, e.g., Peg and Pel. These data demonstrate that *expR_{Ecc}* can, when overexpressed, suppress the production of these virulence determinants. These results are consistent with previous work with the *carR* gene of the *E. carotovora* subsp. *carotovora* strain GS101, encoding a LuxR homologue. CarR acts as a transcriptional activator of antibiotic production in this particular strain. The *carR* gene is not linked to *carI*, which directs the synthesis of OHHL in GS101 (McGowan et al. 1995). When *carR* was introduced on a multicopy plasmid into another strain, SCRI193, that does not produce antibiotics, it was found to reduce the levels of Pel (McGowan et al. 1995), in accordance with our results overexpressing *expR_{Ecc}*. This effect could, in both cases, be partially remedied by addition of exogenous OHHL. This suggests that high levels of ExpR_{Ecc} (and CarR) drain the pool of OHHL possibly needed by other regulatory protein(s), thereby interfering with their activity, i.e., mimicking the effect of an *expI* mutation.

It is interesting to note that purified ExpR_{Ecc} from *E. chrysanthemi* binds directly to the promoters of the *pel* genes,

Fig. 4. ExpR_{Ecc} affects the level of *Erwinia* autoinducer (OHHL). SCC3193 is the wild-type strain and SCC5003 is an *expR_{Ecc}* mutant strain. **A**, Levels of OHHL in the supernatant were measured during growth of *E. carotovora* subsp. *carotovora* in L medium with 0.4% polygalacturonic acid (PGA). Squares represent SCC3193, triangles represent SCC5003, and circles represent the *expI* mutant strain SCC3065, unable to produce OHHL (Pirhonen et al. 1991, 1993), which serves as a negative control. Standard deviation is indicated. **B**, Effect of OHHL addition on suppression of pectate lyase (Pel) production by multiple *expR_{Ecc}* copies (pSA018). Samples were taken from cells grown for 8 h to an OD₆₀₀ of about 3. SCC3065 is an *expI* mutant strain (Pirhonen et al. 1991, 1993), unable to produce OHHL, and serves here as a control. Cells were grown in L medium with 0.4% PGA. Addition of OHHL in **B** was done after 3 h of growth (OD₆₀₀ of about 0.4). Pel activity was measured from culture supernatants.

Table 1. Effect of *expR_{Ecc}* on *expI* expression

Strain ^a	Genotype	GUS activity ^b		
		2 h	6 h	10 h
SCC3193	Wild type	62	161	348
SCC3065	<i>expI</i>	41	185	413
SCC5003	<i>expR_{Ecc}</i>	51	155	343

^a Expression of a plasmid-borne *expI-uidA* transcriptional fusion was characterized in the strains indicated.

^b β-Glucuronidase (GUS) activity expressed as nmol product liberated per min per OD₆₀₀. Cells were grown in L medium containing 0.4% polygalacturonic acid (PGA) and harvested at times indicated. Results shown are from a representative experiment.

among them one of the *pel* genes from *E. carotovora* subsp. *carotovora* (Nasser et al. 1998). The affinity was shown to vary between the various *pel* genes but the binding could be relieved by addition of autoinducer (OHHL). Interestingly, a protected region has also been found in the promoter region of the *E. chrysanthemi* *expI* gene after addition of purified ExpR_{Ech} , but only when OHHL was added (Reverchon et al. 1998). However, an expR_{Ech} mutant of *E. chrysanthemi* did not show any clear phenotype and no significant alteration in Pel production, compared with the wild-type strain.

The most significant effect of an expR_{Ecc} mutation that we have been able to find so far is the increased level of OHHL during early exponential growth. The finding that there is no difference in the expression of a plasmid-borne transcriptional *expI-uidA* fusion between the wild type and the expR_{Ecc} mutant strain and the fact that the level of *expI* transcript was not significantly decreased when expR_{Ecc} was provided in *trans* suggest that ExpR_{Ecc} does not regulate *expI* transcription. Interestingly, the expression of *expI-uidA* was unaffected in the *expI* mutant SCC3065, suggesting that *expI* is not autoregulated. This is in agreement with Nasser et al. (1998), who found that *expI* expression in *E. chrysanthemi* was not altered by the addition of OHHL. Similarly, the *expI* homologue *yenI* in *Yersinia enterocolitica* is not autoregulated and *yenI* was found to be constitutively expressed throughout the growth cycle (Throup et al. 1995).

Recently, it was shown for the first time that a LuxR homologue, EsaR, in *P. stewartii* subsp. *stewartii* functions as a repressor of virulence factor production in this plant pathogen (von Bodman et al. 1998). It has been suggested that the repressor subfamily (EsaR) of LuxR regulators should bind to their target promoters and inhibit transcription in the absence of OHHL (Fuqua et al. 1996; von Bodman et al. 1998). This repression could then be relieved by the action of autoinducer, e.g., by its binding to EsaR. The release of the putative EsaR-OHHL complex from the promoter would allow activation of the target gene. To test whether a similar mechanism exists in *E. carotovora* subsp. *carotovora*, we constructed an *expIR* double mutant. Although there was a slight increase in *pehA* expression, this mutant was found to have a phenotype similar to that of the avirulent *expI* mutant (Pirhonen et al. 1991, 1993). These results indicate that ExpR_{Ecc} -mediated repression of the extracellular enzyme encoding genes in the absence of OHHL cannot be the main reason for the reduced production of virulence determinants in the *expI* mutant. However, we cannot rule out that ExpR_{Ecc} under certain conditions binds to the promoter regions of the extracellular enzyme encoding genes similarly to the *E. chrysanthemi* ExpR_{Ech} protein (Nasser et al. 1998), although in that case it probably plays a minor role in the direct regulation of these genes.

Although our data suggest a negative regulatory role for ExpR_{Ecc} , the relatively modest phenotypic effects observed in expR_{Ecc} mutants indicate that ExpR_{Ecc} is only one of the growing number of regulatory proteins that have been found to modulate virulence gene expression and control virulence in plant pathogenic bacteria (Andersson et al. 1999a, 1999b; Mukherjee et al. 1998; von Bodman and Farrand 1995; von Bodman et al. 1998). This is supported by the finding that a clear phenotype is missing when the expR_{Ech} homologue in *E. chrysanthemi* is inactivated (Nasser et al. 1998). It is also possible that the system is redundant, and there are additional

ExpR -like regulators. Consequently, inactivation of one of these would only cause a minor effect in the phenotype. Finally, we cannot exclude the possibility that ExpR_{Ecc} could also control cellular processes other than virulence.

We are currently exploring the interactions between the different regulatory proteins of *E. carotovora* subsp. *carotovora* and how this regulatory circuitry is used to control virulence of this plant pathogen. It seems clear that other regulators, directly or indirectly involved in quorum sensing, must exist in *E. carotovora* subsp. *carotovora*. Since the isolation of *luxI* homologues can be done rather easily based on their ability to synthesize homoserine lactone, the *luxR* homologues linked to the *luxI*-like genes have been the first ones to be identified and characterized. Therefore, we might so far have missed other important regulators involved in this process.

MATERIAL AND METHODS

Bacterial strains and plasmids.

Escherichia coli host strains used for plasmid maintenance and DNA preparation were DH5 α (Hanahan 1983) and XL1-blue. The latter was purchased from Stratagene (La Jolla, CA). The *E. coli* strain C600 was used for selective labeling of the plasmid-encoded proteins (Tabor and Richardsson 1985). The *E. carotovora* subsp. *carotovora* wild-type strain SCC3193 (Pirhonen and Palva 1988) and the *expI* mutant strain SCC3065 (Pirhonen et al. 1991, 1993) have been described. Plasmids pTK806-1 and pTK806-6 have been described by Pirhonen et al. (1993) and plasmids pHP45 Ω -Cm and pGP1-2 were described by Fellay et al. (1987) and Tabor (1990), respectively. Plasmid pHV200I has been described by Pearson et al. (1994). Transfer of plasmids was by a standard transformation technique or by electroporation with a Bio-Rad gene pulser (Bio-Rad, Richmond, CA). Alternatively, transduction was used as described previously (Pirhonen et al. 1991) with the transducing bacteriophage T4GT7 (Wilson et al. 1979).

Media, culture conditions, and chemicals.

E. coli was propagated in L medium (Miller 1972) at 37°C and *E. carotovora* subsp. *carotovora* at 28°C. Chloramphenicol (Cm) and kanamycin (Km) were added to media at 25 $\mu\text{g/ml}$ and ampicillin (Amp) at 150 $\mu\text{g/ml}$ when required. Cm and Km were not used during the collection of samples for Northern blots and enzyme assays. Polygalacturonic acid (PGA, P-1879; Sigma, St. Louis, MO) was added to a final concentration of 0.4% to induce extracellular enzyme production. The sequencing kit was from USB (United States Biochemicals, Cleveland, OH); the multiprimer DNA labeling kit and radiolabeled compounds were from Amersham International (Buckinghamshire, UK).

Genetic techniques.

For the generation of expR_{Ecc} marker-exchange mutants, two constructs carrying the expR_{Ecc} gene interrupted by a Cm resistance gene were made by first digesting the Cm-specific, 3.5-kb interposon fragment of plasmid pHP45 Ω -Cm (Fellay et al. 1987) with *EcoRI* restriction enzyme followed by isolation of the Ω -Cm fragment. This fragment was blunt ended with Klenow and ligated with *Clal* (SCC5002) or *SnaBI* (SCC5003) digested and blunt-ended pTK806-1. The resulting

two plasmid constructs were named pRK14 and pRK15. For marker exchange, SCC3193/pRK14 and SCC3193/pRK15 were grown overnight on Cm selection and left standing at room temperature for 48 h. Recombinants were selected on L-Cm plates and true marker exchange mutants were screened for by loss of the vector marker (Amp). These mutants were further characterized by Southern analysis of isolated and digested chromosomal DNA and probed separately with *expR_{Ecc}* gene-specific and Cm gene-specific probes. The *expI/expR_{Ecc}* double mutant SCC6005 was constructed with the following strategy: The Ω -Cm resistance marker from pHP45 Ω -Cm (Fellay et al. 1987) was cut out from the plasmid with *HindIII*. The resulting fragment was blunt ended and inserted into pTK806-1 (Pirhonen et al. 1993) that been restricted with *EcoRI/ClaI* and blunt ended. This creates a deletion of about 800 bp in pTK806-1 that spans almost the entire *expI* gene and about 325 bp of *expR_{Ecc}* (see Figure 1A). From this construct the fragment containing the Ω -Cm gene with remaining flanking parts of the *expR_{Ecc}* and *expI* genes was cut out with *XbaI*, blunt ended, and cloned into mini-Tn5 Km1, previously cut with *EcoRI/XbaI* to delete the Km resistance gene. The resulting plasmid was mobilized by conjugation from *E. coli* S17-1 λ pir into *E. carotovora* subsp. *carotovora* SCC3193 as described (de Lorenzo and Timmis 1994). Transconjugants were selected as described (Andersson et al. 1999b). Desired mutations were then confirmed by Southern blot. The *expI-uidA* fusion was constructed as follows: a promoterless *E. coli uidA* gene was amplified by polymerase chain reaction (PCR) and cloned into pBR322. The *expI* promoter region (353 bp upstream of the ATG in the *expI* ORF) was amplified by PCR with primers 5'-AACTGAGCTC TATGCTGAAA CAGGCTTGCT TTCTTC-3' and 5'-CCGACAGTAG TGTGAAGCTT ACATCG-3' and cloned in front of *uidA*. This construct was then confirmed by sequencing. Plasmid pSA018, which contains the *expR_{Ecc}* gene in pBluescript II, was constructed by amplifying *expR_{Ecc}* including the promoter region by PCR with the primers 5'-TTTGGTACCT GATTAATCTC GCTTAATTAG CATT-3' and 5'-AAAGAGCTCC TATGACTGAA CCGGTCGGAT A-3' and chromosomal DNA from SCC3193 as a template. The 3' primer used ended at the translational stop codon of *expR_{Ecc}*. The construct for the controlled *ExpR_{Ecc}* overproduction was done as follows: two synthetic 33-mer oligonucleotides primers (5'-TCTCCAGGAT CCATGTCGCA GTTATTCTAC AAC-3' and 5'-ACAGAAAAGC TTCTATGACT GAACCGGTCG GAT-3') were used to amplify *expR_{Ecc}* from the chromosome of SCC3193. The resulting 737-bp PCR product was cloned as a *BamHI-HindIII* fragment into the corresponding sites of the pQE-30 expression vector (Qiagen, Hilden, Germany) to obtain pRA801. In this vector system, expression is driven from the bacteriophage T5 promoter under control of the *lac* operator. Plasmid pRA801 was transformed into cells that already harbored the pREP4 plasmid (Qiagen) encoding the LacI repressor protein. All PCRs were performed with the proofreading polymerase *Pfu* (Stratagene).

DNA manipulation, deletion analysis, and determination of the nucleotide sequence.

Unless otherwise stated, isolation of plasmid DNA or chromosomal DNA, cloning, and gel analysis of plasmid DNA were by established procedures as described in Sam-

brook et al. (1989). Unidirectional deletions of pTK806-1 were generated by cleaving plasmids with appropriate restriction enzymes within the multiple cloning site to create 3' or 5' overhangs, followed by digestion of the 3' recessed strand with exonuclease III (Henikoff 1984) with the pBluescript II Exo III/Mung Bean DNA deletion kit (Stratagene). Single-strand DNA was removed with mung bean nuclease and blunt ends were ligated and plasmids transformed into *E. coli* XL1-blue strain. Sequencing was performed on both strands by the Sanger dideoxy-chain termination method (Sanger et al. 1977). Amino acid alignments with deduced amino acid sequences were done according to the Needleman-Wunsch algorithm with the PC/GENE package (Oxford Molecular, Oxford).

Isolation of chromosomal DNA and total RNA, and Southern and Northern blot analyses.

Chromosomal DNA was isolated essentially as described by Sambrook et al. (1989) and RNA was isolated by the RNeasy kit (Qiagen). For Southern analysis, chromosomal DNA was digested with restriction enzymes and run on an 0.8% agarose gel. Northern analysis was done essentially as described (Eriksson et al. 1998) with 5 μ g of total RNA and probes specific for *expR_{Ecc}*, *expI*, *pehA*, *pelB*, and *pelC*. A *HindIII-HindIII* interposon fragment from pHP45 Ω -Cm was used as a probe for the Cm resistance gene. Probe fragments were labeled with ³²P-dATP by random primer labeling.

Overproduction of *ExpR_{Ecc}*.

Selective labeling of the *ExpR_{Ecc}* protein was achieved with the T7 RNA polymerase/promoter system essentially as described by Tabor (1990) from the *expR_{Ecc}* gene construct on pTK806-6 (Pirhonen et al. 1993). Labeled proteins were separated by SDS-PAGE and the proteins were visualized by autoradiography of dried gels previously stained with Coomassie brilliant blue. For controlled overexpression of *expR_{Ecc}*, the *E. carotovora* subsp. *carotovora* wild-type strain SCC3193 harboring both pRA801 and pREP4 plasmids was grown overnight in L medium containing Amp, Km, and 2% of glucose. The overnight culture was diluted 1:50 into fresh medium without glucose, containing the appropriate antibiotics and 0.4% PGA, grown for 4 h. Expression of *expR_{Ecc}* was induced in these mid-logarithmic cultures by addition of IPTG to a final concentration of 2 mM. Cultures were grown in the presence of IPTG for 8 h and samples were collected every second hour.

Assay procedures.

Extracellular enzyme indicator plates (Cel, Peh, Pel, and Prt) were as in Pirhonen et al. (1993) and Peh and Pel assays as in Pirhonen et al. (1991). OHHL was measured in a bioassay as follows. Ten microliters of supernatant was diluted with L medium and mixed with 100 μ l of sensor strain (*E. coli* DH5 α harboring the pHV200I⁻ plasmid). Emission of light was measured during a time curve (0 to 2 h), every 30 min with the 1420 multilabel counter VICTOR² (Wallac Oy, Turku, Finland). Results were calculated as slopes of relative light units (RLU) and final values were defined as RLU/OD₆₀₀ (RLU $\times$ ml⁻¹ $\times$ 10²/ OD₆₀₀ $\times$ ml⁻¹). Experiments with addition of synthetic OHHL were done as follows: cells were grown for 3 h (OD₆₀₀ about 0.4) in L medium with 0.4% PGA. At

this point, OHHL was added to a final concentration of 0.1, 1, or 5 µg/ml. Incubation was then continued for 5 h, when samples were taken and Pel activity was assayed from culture supernatants. GUS assays were done according to Wilson (1996).

Virulence assays.

Maceration of potato tubers (*Solanum tuberosum* cv. Bintje) was tested as described in Eriksson et al. (1998). Results were analyzed statistically by the Student's *t* test. A *P* value below 0.05 indicates significance at the 95% level whereas a *P* value below 0.01 indicates significance at the 99% level. Virulence on plants, *Nicotiana tabacum* cv. Samsun, was tested by inoculating the plants with 10⁶ CFU and determining the number of viable bacteria as described earlier (Vidal et al. 1998). Assays on potato stems were done as described by McMillan et al. (1993).

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