A Novel Plant Ferredoxin-Like Protein and the Regulator Hor Are Quorum-Sensing Targets in the Plant Pathogen *Erwinia carotovora*

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Quorum sensing (QS), a population-density-sensing mechanism, controls the production of the main virulence determinants, the plant cell-wall-degrading enzymes (PCWDEs) of the soft-rot phytopathogen *Erwinia carotovora* subsp. *carotovora*. In this study, we used random transposon mutagenesis with a *gusA* reporter construct to identify two new QS-controlled genes encoding the regulator Hor and a plant ferredoxin-like protein, FerE. The QS control of the identified genes was executed by the QS regulators ExpR1 and ExpR2 and mediated by the global repressor RsmA. Hor was shown to contribute to bacterial virulence at least partly through its control of PCWDE production. Our results showed that FerE contributes to oxidative stress tolerance and in planta fitness of the bacteria and suggest that QS could be central to control of oxidative stress tolerance. The presence of the FerE protein appears to be rather unique in heterotrophic bacteria and suggests an acquisition of the corresponding gene from plant host by horizontal gene transfer.

Additional keywords: acylhomoserine lactone, AHSL, ExpI, *Pectobacterium carotovorum*.

Bacteria use a cell-to-cell communication system, referred to as quorum sensing (QS), to monitor their population density and to regulate a wide variety of functions accordingly (Fuqua et al. 2001; Waters and Bassler 2005). These include bioluminescence in *Vibrio fischerii* (Eberhard et al. 1981; Engebrecht and Silverman 1984), conjugal plasmid transfer in *Agrobacterium tumefaciens* (Zhang et al. 1993), biofilm formation and virulence in *Pseudomonas aeruginosa* (Parsek and Greenberg 2000; Winson et al. 1995), production of the exopolysaccharide stewartan in *Pantoea stewartii* (Beck von Bodman and Farrand, 1995), and carbapenem antibiotics and plant cell-wall-degrading enzymes (PCWDEs) in *Erwinia carotovora* subsp. *carotovora* (also referred to as *Pectobacterium carotovorum*) (Bainton et al. 1992; Jones et al. 1993; Pirhonen et al. 1993).

In contrast to the divergence of QS-controlled processes, the basic QS system is similar in many gram-negative bacteria. QS is achieved by the production and sensing of diffusible signal

molecules, *N*-acylhomoserine lactones (AHSL), synthesized by an AHSL synthase, an enzyme of the LuxI family and recognized by a QS regulator, a transcription factor of the LuxR family that in turn controls the expression of downstream genes (Fuqua et al. 2001; Whitehead et al. 2001). Different bacteria produce AHSL with diverse acyl side chain lengths (4 to 16 carbons and with alterations in the oxidative status) that are recognized by a QS regulator specific for the cognate AHSL. Depending on the strain, *E. carotovora* subsp. *carotovora* produces mainly either 3-oxo-hexanoylhomoserine lactone (3-oxo-C6-HSL) or 3-oxo-octanoylhomoserine lactone (3-oxo-C8-HSL) as the major AHSL, with 3-oxo-C8-HSL as the cognate AHSL of *E. carotovora* subsp. *carotovora* SCC3193 (Brader et al. 2005). Normally, the QS regulator is unable to respond to noncognate AHSL. This confers a high degree of specificity to the QS system and suggests that QS is mainly involved in intraspecies communication (Fuqua and Greenberg 2002; Lazdunski et al. 2004; Pappas et al. 2004). The QS regulators are structurally similar, with a ligand (AHSL)-recognizing domain and a conserved DNA-binding domain. Although proteins of the LuxR family have similar structures, their operative mechanisms vary, with some acting as activators and others as repressors. Additionally, some QS regulators bind to DNA in an AHSL-bound form (Pappas et al. 2004; Qin et al. 2000; Whitehead et al. 2002), whereas others bind to DNA and activate transcription in an AHSL-free form, as shown for the EsaR of *Pantoea stewartii* (Minogue et al. 2002, 2005) and the ExpR proteins of *E. carotovora* subsp. *carotovora* (Cui et al. 2006; Sjöblom et al. 2006).

The QS regulators of *E. carotovora* subsp. *carotovora* (ExpR1, ExpR2, ExpR_{Ecc71}, and VirR) have recently been described more extensively. Many *E. carotovora* subsp. *carotovora* strains characterized appear to possess two ExpR proteins (Burr et al. 2006; Cui et al. 2006; Sjöblom et al. 2006). Interestingly, we could show that the two ExpR proteins of SCC3193 have different ligand-binding specificities, with the ExpR1 protein recognizing only the cognate AHSL (3-oxo-C8-HSL) whereas the ExpR2 protein recognized both cognate and noncognate (e.g., 3-oxo-C6-HSL) AHSL (Sjöblom et al. 2006). In the absence of AHSL, the two ExpR proteins function as negative virulence regulators, as indicated by restoration of virulence to the avirulent *expI* mutant by inactivating both *expR1* and *expR2* (Sjöblom et al. 2006). However, the negative control of virulence by the ExpR proteins is not direct but mediated through the global negative regulator RsmA, an RNA-binding protein (Chatterjee et al. 1995, 2005; Cui et al. 2006; Sjöblom et al. 2006). In the AHSL free state, the ExpR1 and ExpR2 proteins bind to the *rsmA* promoter, activating its tran-

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scription and leading to a suppressed virulence. On the other hand, accumulation of the cognate AHSL releases binding of both ExpR proteins and the *rsmA* expression is downregulated, allowing expression of downstream genes such as those for PCWDEs, enhancing virulence (Cui et al. 2006; Sjöblom et al. 2006). The addition of the noncognate AHSL releases only the ExpR2 protein, showing that ExpR1 alone is sufficient to activate *rsmA* transcription and downregulate the downstream genes (Cui et al. 2006; Sjöblom et al. 2006).

Recent transcriptome and proteome analysis have expanded our knowledge of the QS regulons of different gram-negative bacteria (Chen et al. 2003; Qin et al. 2007; Schuster et al. 2003; Wagner et al. 2003). These studies reveal that, in many cases, QS is a global regulatory system affecting a diverse set of both physiological and metabolic processes (e.g., in *Pseudomonas aeruginosa*, over 400 genes are controlled by QS). QS also regulates or fine tunes other regulatory circuits, such as the Rap regulator in *Serratia*. Rap (a homolog of Hor in *Erwinia* spp.) controls the production of the secondary metabolite prodigiosin (Thomson et al. 1997).

In *E. carotovora* subsp. *carotovora* that causes soft rot in a variety of economically important crops worldwide (Perombelon 2002), QS is a central regulator of the main soft-rot-causing determinants, the PCWDEs, including cellulases,

polygalacturonases, pectinases, and proteinases (Barnard and Salmond 2007; Jones et al. 1993; Pirhonen et al. 1991, 1993). *E. carotovora* subsp. *carotovora* mutants deficient in the AHSL synthesizing enzyme ExpI are impaired in the production of PCWDEs and, consequently, are avirulent (Jones et al. 1993; Pirhonen et al. 1993). Furthermore, in some strains of *E. carotovora* subsp. *carotovora*, QS has been shown to control also the production of carbapenem antibiotics (Bainton et al. 1992; McGowan et al. 1995) and to regulate the virulence factors Svx, a secreted homologue of AvrXca of *Xanthomonas campestris* (Corbett et al. 2005), and Nip, an elicitor of plant necrosis (Pemberton et al. 2005).

The aim of this study was to further explore the QS regulon in *E. carotovora* subsp. *carotovora*. Using a transposon mutant screen, we identified two new QS-controlled genes in *E. carotovora* subsp. *carotovora*: a gene encoding a putative plant ferredoxin-like protein and another encoding the regulator Hor. We show that both of these target genes are negatively regulated by ExpR1 and ExpR2 in the absence of AHSL. This regulation appears to be largely mediated through the global negative regulator RsmA. Interestingly, we could show that QS controls oxidative stress tolerance in *E. carotovora* subsp. *carotovora* and that the QS-controlled plant ferredoxin-like protein contributes to this tolerance. The presence of the plant ferredoxin-like gene in *E. carotovora* subsp. *carotovora* points to a potential horizontal gene transfer from a eukaryotic host.

RESULTS

A plant ferredoxin-like protein and the regulator Hor identified as QS targets in a transposon mutagenesis screen.

Previously, we have shown that, in *E. carotovora* subsp. *carotovora* SCC3193, the main virulence determinants, PCWDEs, are controlled by QS (Pirhonen et al. 1993). Further, we have demonstrated that this control is mediated by the global negative regulator RsmA, a target of the QS regulators ExpR1 and ExpR2 in *E. carotovora* subsp. *carotovora* SCC3193 (Sjöblom et al. 2006). To identify new QS-regulated genes, we employed random transposon mutagenesis by introducing the mini-Tn5Cm^r::*gusA* transposon into the AHSL negative *expI* mutant (SCC3065, *expI*::Km^r) (Pirhonen et al. 1993). To identify genes responsive to AHSL, approximately 10,000 Km^rCm^r transposon mutants were screened for β -glucuronidase (GUS) activity with or without 1 μ M 3-oxo-C6-HSLs. From 70 putative mutants picked for further testing, seven were analyzed by quantitative GUS assays. The quantitative GUS assay revealed that two of the mutant strains, SCC802 and SCC804, were clearly responsive to the cognate 3-oxo-C8-HSL of SCC3193 (Fig. 1A). For further studies and to elucidate the expression level of the newly identified QS candidate targets, the mutations were transduced to wild-type SCC3193 background. The AHSL-induced GUS activity of the single mutants SCC802-1 and SCC804-1 was shown to be similar to that of SCC802 and SCC804 double mutants (Fig. 1A). To explore the QS dependence of the mutants and requirement for endogenous AHSL production, GUS activity was assayed (Fig. 1B). These results show that, in the mutant SCC802, the GUS activity is strongly dependent on the presence of *expI*. Similarly, in the SCC804 mutant, the GUS activity responds to the AHSL production, although the basal GUS activity of the SCC804 mutant is higher than that of the SCC802 strain.

To identify the new AHSL-responsive genes tagged by the mini-Tn5Cm^r::*gusA*, the regions flanking the insertion were cloned by arbitrary polymerase chain reaction (PCR) (Caetano-Annoles 1993; O'Toole and Kolter 1998) and sequenced. In the SCC804 mutant, the transposon was inserted into a 438-bp

Fig. 1. A, Cognate *N*-acylhomoserine lactone (AHSL), 3-oxo-octanoylhomoserine lactone (3-oxo-C8-HSL), is needed for full expression of the newly identified quorum-sensing (QS) target genes. β -Glucuronidase (GUS) activity was determined for *Erwinia carotovora* subsp. *carotovora* strains SCC804 and SCC802 grown to an optical density at 600 nm (OD₆₀₀) of approximately 2.8, in L medium without AHSL (open bars), with 1 μ M 3-oxo-hexanoylhomoserine lactone (dotted bars), or with 1 μ M 3-oxo-C8-HSL (striped bars) added. In addition, GUS activity was determined for *E. carotovora* subsp. *carotovora* strains SCC804-1 and SCC802-1 (black bars) (corresponding single mutants with the transposon insertion transferred to wild-type background) grown in L medium to OD₆₀₀ of approximately 2.8. **B,** QS dependence of the target gene expression was assessed by analyzing the GUS activity of the mutants SCC802 (open squares), SCC802-1 (closed squares), SCC804 (open circles), and SCC804-1 (closed circles) at different growth phases. Bars represent standard deviations.

open reading frame (ORF) encoding a putative Hor (homolog of Rap) protein of 146 amino acids (aa) (from here on designated as Hor). The transposon was inserted into *hor* at a position corresponding to amino acid 40. The putative Hor protein of *E. carotovora* subsp. *carotovora* shows 99% identity (99% similarity) to the corresponding protein in the *E. carotovora* subsp. *carotovora* GS101, 98% identity (99% similarity) to the Hor protein of *E. carotovora* subsp. *atroseptica* SCRI1043, 83% identity (92% similarity) to *Serratia* sp. ATCC39006 Rap protein (regulation of antibiotics and pigments), and 75% identity (87% similarity) to a *Yersinia enterocolitica* subsp. *enterocolitica* 8081 protein of the MarR/SlyA-transcriptional regulator family (Fig. 2A). The *hor* locus from *E. carotovora* subsp. *carotovora* SCC3193 with flanking genes was characterized by DNA sequencing (Fig. 2B). This analysis suggests that *hor* is a single gene and not part of an operon.

Interestingly, in the SCC802 mutant, the transposon was inserted into an ORF of 294 bp (the transposon insertion in *ferE* was at a position corresponding to amino acid 70), encoding a putative ferredoxin protein of 97 aa (from here on designated as FerE) most similar to cyanobacterial and plant ferredoxins but with only a limited similarity to ferredoxins from other bacterial genomes (Fig. 3A). An analysis of the *ferE* locus revealed that the closest protein-encoding sequences reside more than 800 bp both upstream and downstream from the FerE encoding gene, suggesting that the *ferE* is not within an operon and, therefore,

will not have polar effects on the transcription on the surrounding genes (Fig. 3B). The exclusive similarity of FerE polypeptide to corresponding plant and cyanobacterial proteins suggests that the *ferE* gene of *E. carotovora* subsp. *carotovora* SCC3193 has been acquired through horizontal gene transfer (HGT). The following evidence support the acquisition of *ferE* by HGT, potentially from an eukaryotic host. i) The highest amino acid sequence identity to any other known protein described in bacteria, except for cyanobacteria and *A. succinogenes*, is less than 30% to a phenylacetate-CoA oxygenase/reductase, PaaK subunit, of *Escherichia coli* B (EAY48515.1). ii) The closest homologues in plants are expressed sequence tags encoding for ferredoxins of *Ipomopsis aggregata* (DT575297.1) and soybean (BU764558.1) that show 64 and 61% identity to FerE, respectively. iii) The G+C content of the *ferE* gene is lower (37%) than the overall G+C content (50.9%) found in the genome of *Erwinia carotovora* subsp. *atroseptica*, a close relative of *E. carotovora* subsp. *carotovora* (Bell et al. 2004), and closer to the overall G+C content (43%) of the *Arabidopsis thaliana* genome (Kuhl et al. 2005).

QS regulators ExpR1 and ExpR2 control *hor* and *ferE* expression.

Northern blot analysis was used to explore whether the AHSL response of *ferE* and *hor* is mediated by the QS regulators ExpR1 and ExpR2. In our previous study, we showed that

Fig. 2. A, Deduced amino acid sequence of Hor of *Erwinia carotovora* subsp. *carotovora* SCC3193 and its alignment with other Hor amino acid sequences. The sequences are from *E. carotovora* subsp. *carotovora* SCC3193 (Hor_EU274295), *E. carotovora* subsp. *carotovora* GS101 (Hor_AAD50820), *Erwinia carotovora* subsp. *atroseptica* (Eca) SCRI1043 (Hor_CAG74834), *Serratia* sp. strain ATCC39006 (Rap_AAF01153), from *Yersinia enterocolitica* subsp. *enterocolitica* 8081 (MarR/SlyA_YP_001006384). **B,** The *hor* locus of *E. carotovora* subsp. *carotovora* SCC3193. The *hor* gene is shown (in white) together with the flanking genes: *gloA* (81% identical to lactoylglutathione lyase from *E. carotovora* subsp. *atroseptica*), *cph1* (86% identical to a conserved hypothetical protein from *E. carotovora* subsp. *atroseptica*), *pcp* (85% identical to an outer membrane protein of *E. carotovora* subsp. *atroseptica*), and *cph2* (95% identical to a conserved hypothetical protein from *E. carotovora* subsp. *atroseptica*). The alignments were performed using the CLUSTALW program (Thompson et al. 1994) and shaded using the BOX shade program (version 3.21; EMBnet website). The arrows show the direction of the reading frames and the striped arrow indicate that we only have a partial sequence. The complementing fragment is indicated as a line.

the ExpR1 and ExpR2 have different AHSL specificities (Sjöblom et al. 2006). To elucidate whether the ExpR proteins act in the same manner in this context, the wild-type SCC3193 and the mutant strains *expl* (SCC3065), *expl expR1* (SCC6005), *expl expR2* (SCC908), and *expl expR1 expR2* (SCC906) were grown without the addition of any AHSL or in the presence of either 3-oxo-C8-HSL or 3-oxo-C6-HSL, and

the accumulation of *ferE* and *hor* mRNAs was characterized by Northern blot analysis (Fig. 4). Our results show that, in the *expl* mutant lacking the AHSL synthase, the expression of both target genes is downregulated whereas, after addition of the cognate AHSL 3-oxo-C8-HSL, both the *ferE* and *hor* genes are fully expressed. This is in accordance to the results obtained with the GUS assay (Fig. 1). In addition, the Northern blot

Fig. 3. A, Deduced amino acid sequence of FerE of *Erwinia carotovora* subsp. *carotovora* SCC3193 and its alignment with other ferredoxin amino acid sequences. The sequences are from *E. carotovora* subsp. *carotovora* SCC3193 (FerE_EU274296), *Arabidopsis thaliana* (FedA_NP_176291), *Solanum tuberosum* (Fed1_CAC38395), *E. carotovora* subsp. *atroseptica* SCRI1043 (Fdx_YP_051321), *E. carotovora* subsp. *carotovora* SCC3193 (Fdx), *Fischerella* sp. strain PCC7605 (PetF_AAC04840), and *Synechococcus* sp. strain PCC6301 (PetF_BAD80674). The four conserved cysteine residues have been indicated with asterisks. **B**, The *ferE* locus of *E. carotovora* subsp. *carotovora* SCC3193. The *ferE* gene is shown (in white) together with the flanking genes: *dsbA* (40% identity to DsbA oxidoreductase from *Azotobacter vinelandii*) and *tn* (77% identity to a putative transposase YPCD1.93 in *Yersinia pestis* CO92). The alignments were performed using the CLUSTALW program (Thompson et al. 1994) and shaded using the BOX shade program (version 3.21; EMBnet website). The arrows show the direction of the reading frames and the striped arrow indicate that we only have a partial sequence. The complementing fragment is indicated as a line.

Fig. 4. ExpR1- and ExpR2-dependent regulation of *ferE* and *hor*. Northern blot analysis of total RNA from *Erwinia carotovora* subsp. *carotovora* wild-type strain SCC3193 (WT), as well as the *expl*, *expl expR1*, *expl expR2*, and *expl expR1expR2* mutant strains grown in L medium, with no *N*-acylhomoserine lactone (-) or supplemented with 1 μ M 3-oxo-hexanoylhomoserine lactone (C6) or 1 μ M 3-oxo-octanoylhomoserine lactone (C8). Total RNAs were extracted from cells at logarithmic phase of growth (optical density at 600 nm = 1.2). RNA was hybridized with *ferE*, *hor*, and 16S rRNA probes.

analysis reveals that both ExpR1 and ExpR2 have specific roles in the control of *ferE* and *hor* genes. In the *expl* *expR1* mutant, the expression of *ferE* and *hor* is activated with the addition of either 3-oxo-C8-HSL or 3-oxo-C6-HSL whereas, in the *expl* *expR2* mutant, the target gene expression is only activated by physiological concentrations of the cognate 3-oxo-C8-HSL. In the *expl* *expR1* *expR2* triple mutant, both *ferE* and *hor* are unregulated, even without any AHSL addition. This suggests that, in the absence of AHSL, ExpR1 and ExpR2 act as negative regulators of *ferE* and *hor* and that this negative regulation is released by the addition of AHSL. In accordance with our previous studies of AHSL regulation of PCWDEs (Sjöblom et al. 2006), we have shown here that the QS regulators ExpR1 and ExpR2 control expression of *hor* and *ferE* in an AHSL specificity-dependent manner. Taken together, the results demonstrate identification of two novel QS (AHSL)-regulated genes, *ferE* and *hor*, in the *E. carotovora* subsp. *carotovora* SCC3193.

Regulation of *hor* and *ferE* is mediated via the global negative regulator RsmA.

Our results (Fig. 4) demonstrated that, in the absence of AHSL, ExpR1 and ExpR2 are negative regulators of *ferE* and *hor*. However, our results do not show whether this repression is direct or mediated by some other regulator. An involvement of the global negative regulator RsmA in QS of *E. carotovora* subsp. *carotovora* was suggested in our previous study, showing that both ExpR1 and ExpR2 activate transcription of *rsmA* in the absence of AHSL and that expression of *rsmA* was fully prevented by addition of the cognate 3-oxo-C8-HSL (Sjöblom et al. 2006). We explored the role of RsmA in controlling *ferE* and *hor* expression by Northern blot analysis (Fig. 5A) using an *expl* mutant and an *expl* *rsmA* double mutant strain. This analysis revealed that, in the *expl* *rsmA* double mutant, the presence of AHSL is not required for the expression of *hor* and *ferE*, in contrast to the *expl* mutant, where presence of the cognate AHSL is necessary. To further support the role of RsmA as the negative regulator of *ferE* and *hor*, we analyzed the expression of these genes in an *expl* *rsmA* double mutant in the presence and absence of the *expl*⁺ allele, mimicking the phenotype of an *rsmA* single mutant. This presence of the *expl*⁺ allele did not alter the *ferE* or *hor* expression observed in the *expl* *rsmA* double mutant, supporting a repressive role of *rsmA* (Fig. 5B). Taken together, our results suggest that both *ferE* and *hor* are controlled by RsmA, which in turn is controlled by the QS regulators ExpR1 and ExpR2 in an AHSL-dependent manner.

Hor contributes to virulence of *E. carotovora* subsp. *carotovora*.

Previous studies have demonstrated that the QS system controls the production of PCWDEs and, thus, is one of the most important factors regulating virulence in *E. carotovora* subsp. *carotovora*. To elucidate the possible role of the above-identified new QS-controlled genes in virulence, we first characterized the capacity of the corresponding mutants to macerate potato tubers. Potato tuber slices (cv. Van Gogh) were inoculated with the wild-type strain SCC3193 and the *expl*, *hor*, *ferE*, *expl* *hor*, or *expl* *ferE* mutants (Fig. 6B). To ensure that the differences in maceration capacity were due to defects in virulence determinants rather than in the growth properties of the different strains, the growth of the strains was determined in both complete and minimal media (Supplementary Figure S1). These results indicate that the observed differences in maceration cannot be explained by differences in bacterial growth. As expected, the avirulent *expl* mutant showed almost no maceration. Similarly, the *expl* *ferE* and *expl* *hor* double mutants were

not able to macerate potato tubers. The *hor* single mutant showed substantially reduced capacity of maceration, while the *ferE* mutant was similar to the wild type. The impaired maceration capacity of the *hor* mutant is in accordance with its reduced production of PCWDEs as assessed by enzyme indicator plates (Fig. 6A). In addition, we showed that the reduced PCWDE production of the *hor* mutant could be complemented by expressing the *hor* gene in trans. To further assess the virulence of the *hor* and *ferE* mutants, leaves of *A. thaliana* Col-0 plants were inoculated with the wild-type strain SCC3193, the *expl* mutant, the *hor* mutant, or the *ferE* mutant. These results were similar to those of the potato maceration assay, with the *expl* mutant showing almost no maceration, the *hor* mutant showing clearly reduced maceration, and the *ferE* mutant and the wild-type showing similar level of maceration of leaf tissue (Fig. 6C). Taken together, the results clearly suggest that the Hor regulator contributes to the virulence and development of disease symptoms by the *E. carotovora* subsp. *carotovora* SCC3193.

To investigate the possible role of Hor and FerE in the infection process, we measured the in planta growth of the respective mutants in *A. thaliana* Col-0. This analysis revealed that both the *hor* and the *ferE* single mutants showed slightly reduced growth compared with the wild-type SCC3193, although the reduction was not as clear as with the avirulent *expl* mutant (Fig. 6D). This suggests that both Hor and FerE contribute to the overall fitness of the bacteria during infection.

QS regulates oxidative stress tolerance in the plant pathogen *E. carotovora* subsp. *carotovora*.

The *ferE* gene of *E. carotovora* subsp. *carotovora* encodes a plant ferredoxin-like protein present in plants and photo-

Fig. 5. Quorum-sensing control of *ferE* and *hor* is mediated by RsmA. **A**, Northern blot analysis of total RNA from *expl* and *expl* *rsmA* mutant strains grown in L medium with no *N*-acylhomoserine lactone (–) or supplemented with either 1 μ M 3-oxo-hexanoylhomoserine lactone (C6) or 1 μ M 3-oxo-octanoylhomoserine lactone (C8). **B**, Northern blot analysis of total RNA from *expl* and *expl* *rsmA* mutant strains with *expl*_{SCC3193} expressed in trans; “c” indicates vector control pBluescript SK+. Total RNAs were extracted at logarithmic phase of growth (optical density at 600 nm = 1.2). RNA was hybridized with *ferE*, *hor*, and 16 S *rRNA* probes.

synthetic cyanobacteria (Fig. 3A). The *ferE* gene of *E. carotovora* subsp. *carotovora* SCC3193 appears to be a novel acquisition in heterotrophic bacteria sequenced up to today. The FerE protein is most similar to ferredoxins PetF (BAD80674) of *Synechococcus elongatus* PCC 6301 (62% identity and 79% similarity) and PetF (AAC04840) of *Fischerella* subsp. PCC7605 (64% identity and 76% similarity); to plant ferredoxins, including FedA (NP_176291) of *A. thaliana* (56% identity and 76% similarity) and FedI (CAC38395) of *Solanum tuberosum* (58% identity and 73% similarity) (Fig. 3A); and to putative plant ferredoxins from *I. aggregata* (63% identity and 75% similarity to DT575297.1) and soybean (61% identity and 73% similarity to BU764558.1). FerE of *E. carotovora* subsp. *carotovora* showed only very limited similarity to a putative bacterial ferredoxin Fdx (26% identity and 48% similarity) in the se-

quenced genome of *E. carotovora* subsp. *atroseptica* (Fig. 3A). Moreover, a putative Fdx protein was also found in the *E. carotovora* subsp. *carotovora* SCC3193 with an amino acid sequence almost identical (99% identity and 100% similarity) to the *E. carotovora* subsp. *atroseptica* Fdx sequence (unpublished). The presence of the plant-type ferredoxin found here suggests that it may have a separate role from the Fdx of *E. carotovora* subsp. *atroseptica* and *E. carotovora* subsp. *carotovora*.

The FerE of SCC3193 contains a conserved iron-sulfur-binding domain 2Fe-2S, which plays an important role in electron transfer processes and in various enzymatic reactions (Marchler-Bauer et al. 2007). In *Escherichia coli* and *Erwinia chrysanthemi*, proteins with iron-sulfur clusters have been shown to have an effect on the bacterial tolerance to oxidative stress (Koo et al. 2003; Ollagnier-de Choudens et al. 2003). To explore whether the FerE of SCC3193 is involved in oxidative stress tolerance, we assessed the susceptibility of the wild-type strain SCC3193, the *expl* mutant, and the *ferE* mutant to H₂O₂. These strains were exposed to 20, 30, and 40 mM H₂O₂ and their survival rate was compared with that of the wild-type strain SCC3193 (Fig. 7A). Our results demonstrate a clear concentration-dependent reduction in oxidative stress tolerance in the *ferE* mutant strain. This is in contrast to the *hor* mutant that exhibited wild-type levels of oxidative stress tolerance (data not shown). These results show for the first time that, in the plant-pathogenic *E. carotovora* subsp. *carotovora*, the oxidative stress tolerance is partly mediated via FerE. Further, our data show that the AHL-deficient *expl* mutant is even more sensitive to oxidative stress, with 40 mM H₂O₂ concentration being lethal for this mutant. This deficiency in oxidative stress tolerance in the *expl* mutant could be complemented with the addition of 1 μ M 3-oxo-C8-HSL (Fig. 7B). To confirm the contribution of FerE to the oxidative stress tolerance, we expressed *ferE* in trans in both the *ferE* mutant and the *expl* mutant (Fig. 7C). This experiment demonstrated that the reduced oxidative stress tolerance of the *ferE* mutant was complemented by the addition of the *ferE*⁺ in trans. Introduction of the *ferE*⁺ clone to the *expl* mutant partially restored the oxidative stress tolerance of the *expl* mutant. Taken together, our results suggest that, in the plant pathogen *E. carotovora* subsp. *carotovora*, QS controls oxidative stress tolerance and that the cognate 3-oxo-C8-HSL is required for this tolerance. Moreover, it shows that FerE contributes to this QS-controlled oxidative stress tolerance.

Fig. 6. Role of *hor* and *ferE* in virulence. **A**, Exoenzyme indicator plates showing the production of cellulases (Cel) and pectinases (Peh) from 10- μ l culture supernatant of the following strains: wild-type SCC3193 (WT), *expl* mutant, *ferE* mutant, *hor* mutant, and *hor* mutant with *hor* expressed in trans. **B**, Potato tuber slices were inoculated with the following *Erwinia carotovora* subsp. *carotovora* strains: WT SCC3193, *expl* mutant, *ferE* mutant, *hor* mutant, *expl ferE* double mutant, and *expl hor* double mutant using a bacterial inoculum of 10⁵ CFU. At 72 h after inoculation, the macerated tissue of the potato was weighed to determine the maceration capacity. **C**, Leaves of *Arabidopsis thaliana* accession Col-0 were locally inoculated with the same strains as in A using an inoculum of 10⁴ CFU and the symptoms were documented after 48 h. Representative leaves of the experiment are shown. **D**, To determine the bacterial growth in planta of the above *E. carotovora* subsp. *carotovora* strains in *A. thaliana* Col-0, plant samples were taken at 48 h post inoculation and the amount of living bacteria was determined according to Materials and Methods. The result shows an average of six infected plants. Different letters at data points indicate significant differences ($P < 0.05$) calculated with data of all replicates at each time point with one-way analysis of variance and Tukey's honestly significantly different test. Bars represent standard deviations.

DISCUSSION

QS is central to virulence regulation in the soft-rot plant pathogen *E. carotovora* subsp. *carotovora*. In previous studies, we have shown that the production of PCWDEs, which are the main virulence determinants and required for plant tissue maceration, is repressed in the *expI* mutant lacking the AHSL synthase (Pirhonen et al. 1991, 1993). Recent data demonstrate that two QS regulators, ExpR1 and ExpR2, with distinct AHSL specificities are key sensors of AHSL in *E. carotovora* subsp. *carotovora*, and the simultaneous deletion of *expI*, *expR1*, and *expR2* genes leads to a constitutive activation of PCWDE production (Sjöblom et al. 2006).

To explore whether QS could control the expression of additional virulence-related genes in *E. carotovora* subsp. *carotovora*, we employed a random transposon mutagenesis with a mini-Tn5Cm^r::*gusA* transposon in *expI* background. A screen for AHSL-responsive genes identified two new QS-regulated genes which have not been identified in earlier QS screening studies performed in *E. carotovora* subspecies (Corbett et al. 2005; Pemberton et al. 2005). In this study, we present three major findings. First, we demonstrate the identification of two new QS-regulated genes, encoding the regulator Hor and a putative novel plant ferredoxin-like protein, FerE. FerE is similar to cyanobacteria and plant ferredoxins but with very limited similarity to bacterial ferredoxins sequenced up to today. Second, we provide data showing that these newly identified genes are regulated by the QS regulators ExpR1 and ExpR2 and the regulation is, to a large extent, mediated by the negative regulator RsmA. Third, we show that QS controls oxidative stress tolerance in *E. carotovora* subsp. *carotovora* and that FerE is part of this regulon contributing to QS-dependent oxidative stress tolerance.

Northern blot analysis was used to demonstrate how QS regulation of *hor* and the *ferE* is executed. As we have shown previously for PCWDE production (Sjöblom et al. 2006), the QS regulators ExpR1 and ExpR2 seem to repress *hor* and *ferE* expression in the absence of AHSL. This repression is abolished by addition of the cognate 3-oxo-C8-HSL leading to expression of *hor* and *ferE*. In our previous study, we also showed a difference in AHSL recognition specificity between the ExpR1 and ExpR2 proteins, with ExpR1 acting as a cognate ligand-specific regulator and ExpR2 acting as a more promiscuous regulator, responding to both cognate and noncognate signals (Sjöblom et al. 2006). Our results demonstrate that ExpR1 and ExpR2 act in a similar way, controlling *hor* and *ferE* expression. In the *expI expR1* double mutant, transcription of *hor* and *ferE* was induced by both 3-oxo-C6-HSL and 3-oxo-C8-HSL whereas, in the *expI expR2* double mutant, the *hor* and *fer* were induced only by the cognate 3-oxo-C8-HSL (Fig. 8).

However, these data do not demonstrate whether ExpR1 and ExpR2 directly control the newly identified QS target genes or whether the regulation is mediated by some other factor. We and others have recently demonstrated that the ExpR control of PCWDEs is mediated by RsmA (Cui et al. 2006; Sjöblom et al. 2006), an RNA-binding protein that negatively regulates PCWDE gene expression (Chatterjee et al. 2005). Northern blot analysis showed that *hor* and *ferE* expression is indeed controlled in a similar fashion. Our results suggest that, in addition to PCWDE genes, *hor* and *ferE* could be targets of the global negative regulator RsmA in *E. carotovora* subsp. *carotovora* (Fig. 8). Additional RsmA targets in *E. carotovora* subsp. *carotovora* (strain Ecc71) have earlier been shown to include also HrpN_{Ecc} (Cui et al. 1996). In *P. aeruginosa*, RsmA is known to regulate several virulence-related genes, including the rhamnolipid biosynthetic operons, hydrogen cyanide, and

AHSL synthase genes (Heurlier et al. 2004; Pessi et al. 2001). Additionally, a recent transcriptome analysis performed in *P. aeruginosa* with an *rsmA* mutant revealed several potential RsmA target genes (Burrowes et al. 2006). It remains to be elucidated whether all QS regulation in *E. carotovora* subsp. *carotovora* SCC3193 is executed by RsmA or whether there is also an RsmA-independent QS pathway. Furthermore, it remains to be solved whether there are other targets besides *rsmA* regulated directly by either ExpR1 or ExpR2 in *E. carotovora* subsp. *carotovora*.

The two genes of the QS regulon identified in this study represent two different kinds of proteins, a transcriptional regula-

Fig. 7. Role of *ferE* and *expI* in oxidative stress tolerance of *Erwinia carotovora* subsp. *carotovora*. **A**, The wild-type SCC3193 (WT) (gray bars), *expI* mutant (white bars), and *ferE* mutant (dotted bars) strains of *E. carotovora* subsp. *carotovora* were exposed to 20, 30, and 40 mM H₂O₂. The number of living bacteria was measured before the addition of H₂O₂ and after incubating the cells for 4 h with H₂O₂ by viable counts. The survival of the *expI* mutant and the *ferE* mutant was given as percentage of the survival of the WT bacteria. **B**, Complementation of the oxidative stress susceptibility of *expI* mutant by *N*-acylhomoserine lactone (AHSL) addition. The indicated strains were grown in L medium without AHSL or supplemented with 1 μ M 3-oxo-hexanoylhomoserine lactone (C6) or 1 μ M 3-oxo-octanoylhomoserine lactone (C8) and subjected to 40 mM H₂O₂ for 4 h. **C**, For complementation studies, *ferE* was expressed in trans in the *ferE* and *expI* mutant strains. To test the oxidative stress susceptibility, the indicated strains were grown in L medium and subjected to 40 mM H₂O₂ for 4 h; "c" indicates vector control pQE30. Bars represent standard deviations.

tor and an enzyme, which highlights the complexity of the QS regulon and variety of genes controlled by the QS. The complexity is also supported by other screens accomplished in *E. carotovora* (Corbett et al. 2005; Pemberton et al. 2005) and in *P. aeruginosa* (Schuster et al. 2003), where proteins involved in diverse cellular functions have been identified. It is interesting to find *hor* as a QS-controlled gene in *E. carotovora* subsp. *carotovora* SCC3193, whereas previous studies suggested that the *hor* gene is not present in SCC3193 (Thomson et al. 1997) and that *hor* was not under QS regulation in another *E. carotovora* subsp. *carotovora* strain (McGowan et al. 2005). Hor is a member of the MarR/SlyA family of regulatory proteins known to control antimicrobial activities (Thomson et al. 1997). In certain strains of *E. carotovora* subsp. *carotovora*, Hor controls the expression of biosynthetic genes for the antibiotic carbapenem, which are also regulated by QS via the regulator CarR of the LuxR family (Holden et al. 1998; Thomson et al. 1997, 2000). In these strains, Hor and QS control of the same genes seem to work in parallel. However, *E. carotovora* subsp. *carotovora* SCC3193 appears to lack the carbapenem-synthesizing genes (Holden et al. 1998). Our results demonstrate that, in SCC3193, expression of *hor* is controlled in an AHL-dependent manner in contrast to the *E. carotovora* subsp. *carotovora* ATTN10, where Hor regulation is not under QS control (McGowan et al. 2005). Interestingly, in the opportunistic pathogen *Serratia* subsp. ATCC39006, the Hor homolog Rap also regulates carbapenem biosynthesis and, in this case, in a QS-dependent manner (Fineran et al. 2005; Slater et al. 2003). Nevertheless, it remains to be clarified whether Hor of the *E. carotovora* subsp. *carotovora* SCC3193 also has a role in controlling antimicrobial processes as demonstrated for other proteins in the MarR/SlyA family.

To explore other possible functions of Hor in SCC3193, we characterized its role in virulence by both potato tuber maceration and by *Arabidopsis* leaf inoculations. Both assays clearly revealed reduced maceration capacity of the *hor* mutant compared with the wild-type *E. carotovora* subsp. *carotovora*

strain. Furthermore, the *hor* mutant showed reduced bacterial growth in planta. In accordance with the reduced maceration capacity observed, the production of cellulase and polygalacturonase was also somewhat reduced. This is in accordance with previous results showing reduced plant maceration by a *hor* mutant of *E. carotovora* subsp. *carotovora* ATM101 (Thomson et al. 1997). In conclusion, part of QS control of PCWDE genes and, hence, virulence appears to be directed through Hor in SCC3193.

The *ferE* gene identified represents a novel gene encoding a plant ferredoxin-like protein in a heterotrophic bacterium. The corresponding putative FerE protein is clearly distinct from normal bacterial ferredoxins (i.e., those found in other plant pathogens). Although the *E. carotovora* subsp. *atroseptica* genome of strain SCRI1043 possesses at least 10 genes encoding proteins classified as different kinds of ferredoxins (GenBank BX950851), they are clearly distinct from the plant ferredoxin-like protein characterized in this study. The putative FerE protein of SCC3193 has a conserved 2Fe-2S site (Marchler-Bauer et al. 2007) and has high similarity to Ferredoxin I (FedI and PetF) of cyanobacteria (Floss et al. 1997) and to plant ferredoxins of the Fd2 (= FedA) type of *Arabidopsis* (Hanke et al. 2003). These ferredoxins have been implicated to processes such as nitrogen and sulfur assimilation, sulfite reduction, amino acid and fatty acid metabolism, and redox regulation, as well as mediating electron transfer from the photosystem I (PSI) to ferredoxin NADP reductase (Hanke et al. 2003; Mazouni et al. 2003).

It is interesting to find such a QS-regulated gene in the plant pathogen *E. carotovora* subsp. *carotovora*, and its similarity to plant ferredoxins might indicate that the *ferE* gene of *E. carotovora* subsp. *carotovora* SCC3193 has been acquired through HGT. It has been estimated that HGT might contribute to 6 to 21% of the genes of plant-pathogenic bacteria (Garcia-Vallve et al. 2003). Accordingly, analysis of the genome of *E. carotovora* subsp. *atroseptica* indicates a substantial percentage of genes acquired by HGT (Toth et al. 2006). Although most HGT-

Fig. 8. Schematic model for *N*-acylhomoserine lactone (AHL)-dependent regulation of oxidative stress tolerance and virulence by ExpR1 and ExpR2 mediated by RsmA in *Erwinia carotovora* subsp. *carotovora*. In the absence of AHL, both ExpR1 and ExpR2 activate the expression of *rsmA*, negatively regulating oxidative stress tolerance and virulence. After addition of 3-oxo-hexanoylhomoserine lactone (3-oxo-C6-HSL), the ExpR2 is released from the transcriptional activation of *rsmA*, but ExpR1 is still present, activating *rsmA*. In the presence of the cognate 3-oxo-octanoylhomoserine lactone (3-oxo-C8-HSL), both ExpR1 and ExpR2 are released, downregulating *rsmA*. This leads to enhanced expression of *ferE* and *hor*, which results in enhanced oxidative stress tolerance and virulence and prompts bacterial fitness in planta.

derived genes seem to originate from other bacteria, a smaller but still significant number of genes (approximately 1%), particularly in plant and animal pathogens and symbionts, appear to have been acquired by HGT from eukaryotes (Brown 2003; Koonin et al. 2001). The life style of bacterial pathogens is reflected by genes presumably transferred from animals to the animal pathogen *P. aeruginosa* and by an excess of genes potentially derived from plants to the plant pathogen *Xylella fastidiosa* (Koonin et al. 2001). Another example of potential HGT from the plant host to *E. carotovora* subsp. *carotovora* is provided by the *pehA* gene encoding a polygalacturonase that shows striking similarity to a corresponding protein in tomato (Hinton et al. 1990; Saari-lahti et al. 1990).

HGT from a eukaryotic host seems to be a likely event, because HGT probably is facilitated in the phytosphere and in bacteria with a lifestyle closely associated to plants (van Elsas et al. 2003). However, we cannot rule out the possibility that *ferE* of *E. carotovora* subsp. *carotovora* SCC3193 has been acquired by HGT from cyanobacteria with a similar percentage of identity to FerE as in corresponding plant proteins (64% identity to *Fischerella* subsp. PCC7605 ferredoxin PetF; AAC04840).

The putative FerE protein did not appear to be involved in PCWDE production or in the plant tissue maceration in either of the plant assays (Fig. 6B and C). Interestingly, however, the growth rate of *ferE* mutants in *Arabidopsis* was slightly compromised. This suggests that *ferE* could have a role in survival of *E. carotovora* subsp. *carotovora* in planta during infection. The presence in FerE of a conserved iron-sulfur binding cluster, often found in enzymes involved in oxidative stress responses (Outten 2007), prompted us to assess the possible role of FerE of SCC3193 in oxidative stress tolerance. Our results demonstrate a substantially reduced tolerance to H₂O₂ in the *ferE* mutant compared with the wild-type strain. This suggests that FerE has an important role in oxidative stress tolerance of *E. carotovora* subsp. *carotovora*, showing for the first time a biological function for a plant ferredoxin-like protein in heterotrophic bacteria. The observed contribution to oxidative stress tolerance of FerE as well as the reduced in planta growth of the *ferE* mutant suggests that FerE is required for bacterial fitness during the infection process.

To investigate the overall role of QS in the oxidative stress tolerance of *E. carotovora* subsp. *carotovora*, we examined the

H₂O₂ tolerance of the *expI* mutant. The *expI* mutant was even more susceptible to oxidative stress than the *ferE* mutant, showing clearly that, in the plant pathogen *E. carotovora* subsp. *carotovora* SCC3193, QS controls oxidative stress tolerance. Earlier studies in bacteria such as *Pseudomonas* spp. and *Burkholderia pseudomallei* support our results showing an association between oxidative stress tolerance and QS (Hassett et al. 1999; Quiñones et al. 2005; Lumjiaktase et al. 2006). In *Pseudomonas syringae*, QS was suggested to regulate the epiphytic fitness, including traits such as oxidative stress tolerance, EPS production, and swarming motility. The oxidative stress response was shown to be in association with the alginate production (Quiñones et al. 2005). In this study, we have identified a new important factor, FerE, being part of the oxidative stress tolerance regulon. This suggests that QS regulates a diverse set of factors conferring oxidative stress tolerance. The joining of *E. carotovora* subsp. *carotovora* to the group having oxidative stress as one of the QS targets indicates that this stress response could be one of the key traits regulated by QS in general. Oxidative burst play a central role in many host-pathogen interactions; therefore, the role of a cell density-dependent mechanism as a key regulator of oxidative stress tolerance is legitimate.

In conclusion, using a transposon mutagenesis and analysis of selected mutants, we have identified previously new QS-regulated genes in the genus *Erwinia*. This discovery highlights the fact that the cooperative working model of ExpR1 and ExpR2 is not restricted only to the regulation of the main virulence determinants, the PCWDEs, and further deepens our understanding of the impact QS system has on different cellular activities involved in pathogen-host interactions, such as survival of oxidative stress, one of the main host defense systems.

MATERIALS AND METHODS

Bacterial strains and media.

Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were cultured in L medium (Miller 1972) at 37°C and *Erwinia carotovora* subsp. *carotovora* strains at 28°C. As minimal media, M9 media was used (Miller 1972). Chloramphenicol (Cm) was added at 50 µg ml⁻¹ and kanamycin (Km) at 50 µg ml⁻¹ when required and if not

Table 1. Bacterial strains used in this study

Strain or plasmid	Genotype	Reference
Strains		
<i>Escherichia coli</i>		
S17-1 λ-pir/ pRG (pUT-mini-Tn5Cm ^r ::gusA)	Tp ^r Sm ^r <i>recA</i> <i>thi</i> <i>pro</i> <i>hsdR</i> ⁻ <i>M</i> ⁺ RP4:2-Tc:MuKm Tn7 λ pir	de Lorenzo et al. 1990; Marits et al. 1999
<i>Erwinia carotovora</i> subsp. <i>carotovora</i>		
SCC3193	Wild type	Saari-lahti and Palva 1986
SCC3065	<i>expI</i> ::km in SCC3193 background, Km ^r	Pirhonen et al. 1991
SCC6005	<i>expIexpR1</i> ::cm in SCC3193 background, Cm ^r	Andersson et al. 1991
SCC906	<i>expR2</i> ::km in SCC6005 background, Km ^r Cm ^r	Sjöblom et al. 2006
SCC908	<i>expR2</i> ::cm in SCC3065 background, Km ^r Cm ^r	Sjöblom et al. 2006
<i>expI rsmA</i> mutant	<i>ΔrsmA</i> in SCC3065 background, Km ^r	R. A. Andersson (<i>unpublished</i>)
SCC802-1	<i>ferE</i> ::cm <i>gusA</i> in SCC3193 background, Cm ^r	This work
SCC802	<i>ferE</i> ::cm <i>gusA</i> in SCC3065 background, Km ^r Cm ^r	This work
SCC804-1	<i>hor</i> ::cm <i>gusA</i> in SCC3193 background, Cm ^r	This work
SCC804	<i>hor</i> ::cm <i>gusA</i> in SCC3065 background, Km ^r Cm ^r	This work
Plasmids		
pBluescript SK+ (pBSK+)	Cloning vector, Amp ^r	Stratagene
pQE30	Expression vector, Amp ^r	Qiagen
<i>expI</i> _{SCC3193} in pBSK+	<i>expI</i> _{SCC3193} cloned into pBSK+	Brader et al. 2005
pSMS30	<i>ferE</i> cloned into pQE30 <i>Eco</i> RI and <i>Bam</i> HI sites	This work
pSMS31	<i>hor</i> cloned into pQE30 <i>Eco</i> RI and <i>Bam</i> HI sites	This work

^a Tp^r, Sm^r Km^r Cm^r Amp^r indicates resistance to trimethoprim, streptomycin, kanamycin, chloramphenicol, and ampicillin, respectively.

otherwise mentioned. AHSL were used at a concentration of 1 μ M if not otherwise mentioned.

Transposon mutagenesis and construction of mutant strains.

For the generation of *E. carotovora* subsp. *carotovora* double mutants responsive to AHSL, the *expl* mutant (SCC3065) (Table 1) was used as recipient. The donor strain *Escherichia coli* S17-1 λ -pir containing a suicide vector pUT-mini-Tn5Cm^r::*gusA* was used in biparental matings as described by de Lorenzo and associates (1990) and Marits and associates (1999). Transconjugants were selected on L agar plates containing Cm and Km. AHSL-responsive mutants were selected on L agar plates containing XglcA at 50 μ g ml⁻¹, appropriate antibiotics, and with or without 1 μ M 3-oxo-C6-HSL. Replicate plates with or without 3-oxo-C6-HSL were compared to identify colonies with changed color intensity in response to the presence of AHSL. The cognate AHSL for the *E. carotovora* subsp. *carotovora* SCC3193 used in this study was 3-oxo-C8-HSL (Brader et al. 2005). However, the strain responds also to higher concentrations of the related but noncognate 3-oxo-C6-HSL (Brader et al. 2005; Pirhonen et al. 1993). We used the 3-oxo-C6-HSL for the screening instead of the 3-oxo-C8-HSL due to its commercial availability at that time.

The transposon mutants were transferred to wild-type background by generalized transduction and subsequent homologous recombination using T4GT7 phages as described previously (Pirhonen and Palva, 1988; Pirhonen et al. 1991). Northern blot analysis was used to verify that expression of the genes was indeed inactivated by the insertion (data not shown).

DNA techniques.

To identify the site of transposon insertion of the new mutants, we used arbitrary PCR (Caetano-Annoles 1993; O'Toole and Kolter 1998) and subsequent sequencing of the PCR products. In this method, the DNA flanking the insertion site is enriched in two rounds of amplification with primers specific to the ends of the Tn5 element and primers specific to the random sequence, which can anneal to chromosomal sequences flanking the transposon. Subsequently, *ferE* and *hor* probes were designed to screen a Lambda DASH library (Stratagene, La Jolla, CA, U.S.A.), containing 17- to 22-kb fragments of *E. carotovora* subsp. *carotovora* SCC3193, to obtain the flanking regions. *FerE*- and *hor*-positive clones were used for subsequent arbitrary PCR. All of the sequencing reactions were performed by an automatic laser fluorescent sequencer (Pharmacia, Uppsala, Sweden) at the Institute of Biotechnology (University of Helsinki, Finland).

Construction of plasmids.

The *ferE* and *hor* genes were amplified by PCR from wild-type SCC3193 genomic DNA using the primers FerE F compl (5'-CGGAATTCGTTGATTTTCTTATAAATT-3') and FerE R compl (5'-CGGGATCCTTAATATAAATTCTTTTCC-3') and Hor F compl (5'-CGGAATTCATGGAATTGCCATTAGGAT-3') and Hor R compl (5'-CGGGATCCTTACACTTGATTTTCATGT-3'). The 576-bp *ferE* PCR product and the 438-bp *hor* PCR product were digested with *Bam*HI and *Eco*RI and ligated into pQE30, digested with corresponding enzymes, resulting in pSMS30 and pSMS31, respectively.

RNA isolation and Northern blot analysis.

Erwinia cells from overnight cultures were diluted 1/100 in L medium and grown at 28°C. Samples for RNA isolation were taken at indicated time points and growth was monitored by measuring the optical density at 600 nm (OD₆₀₀). Total RNA was isolated as described by Sambrook and associates

(1989). Northern blot analysis was performed with 10 μ g of total RNA separated in 1.5% formaldehyde gel. Specific digoxigenin-labeled DNA fragments for the Northern blots were amplified with PCR using the following primers: *ferE* (Fw) 5'-GCTCATCATCTAAAAAAGACTGATC-3' and (Rev) 5'-GATATAGAGTGCTCCGATGATG-3' and *hor* (Fw) 5'-TGCAATCAGGGATATAAC-3' and (Rev) 5'-GTTATGCAACGTGACC CA-3'; and 16S *rRNA* primers F2115 and F2116 (Hyytiäinen et al. 2003). The blotting hybridization and digoxigenin detection was performed according to the manufacturer's instructions (Roche, Diagnostics, Basel, Switzerland).

Enzyme assays.

Cellulase (Cel) and polygalacturonase (PehA) activities were assayed from 10 μ l of supernatant of overnight-grown liquid cultures on corresponding enzyme indicator plates (Pirhonen et al. 1993). For GUS activity assay, *Erwinia* cells from overnight cultures were diluted 1/100 in L medium and grown at 28°C. Samples for GUS activity assay were taken at indicated time points and the growth was monitored by measuring the OD₆₀₀. GUS activity was measured by using *p*-nitrophenyl β -D-glucuronide as substrate (Marits et al. 2002; Novel et al. 1974).

Oxidative stress tolerance.

To test oxidative stress tolerance cells, H₂O₂ was added directly to an overnight liquid culture to a final concentration of 20, 30, or 40 mM and the cells were incubated for 4 h at 28°C by shaking in the dark. The cells were plated and CFU were counted both before and after the stress treatments (Andersson et al. 1999).

Virulence tests.

To test the maceration capacity, *E. carotovora* subsp. *carotovora* strains were grown overnight and diluted into 0.9% NaCl, and samples containing 10⁵ bacterial cells ml⁻¹ were used for inoculation of 1-cm-thick potato slices (*S. tuberosum* cv. Van Gogh). Infected potato slices were incubated at 28°C for 72 h under humid conditions with wet tissue paper in the incubation box. The amount of soft rot was measured by scraping and subsequently weighing the rotted tissue.

A. thaliana Col-0 plants were grown in 1:1 vermiculite and peat mixture with a 12-h light period at 22°C, and 4-week-old plants were used for experiments. *A. thaliana* Col-0 plants were infected by wounding and local inoculation of approximately 10⁴ CFU bacteria in 0.9% NaCl per leaf. The infected plants were kept in a controlled environment (22°C, 12 h of light, and high humidity) and the development of disease symptoms were documented after 48 h. To determine bacterial growth in planta (CFU/plant) in *Arabidopsis*, the infected plants were homogenized into 0.9% NaCl and serial dilutions of bacteria were plated on L plates with appropriate antibiotics added. Statistical analysis was performed with SPSS 10.07. The data of all replicates were normally distributed and were analyzed by one-way analysis of variance followed by Tukey's honestly significant difference post hoc test for multiple comparisons between the means.

Nucleotide sequence accession number.

The DNA sequence data determined in this study has been submitted to the DDB/EMBL/GenBank databases under accession number EU274296 (*FerE*) and EU274295 (*Hor*).

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