Novel Quorum-Sensing-Controlled Genes in *Erwinia carotovora* subsp. *carotovora*: Identification of a Fungal Elicitor Homologue in a Soft-Rotting Bacterium

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Seven new genes controlled by the quorum-sensing signal molecule *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL) have been identified in *Erwinia carotovora* subsp. *carotovora*. Using *TnphoA* as a mutagen, we enriched for mutants defective in proteins that could play a role in the interaction between *E. carotovora* subsp. *carotovora* and its plant hosts, and identified Nip_{Ecc} and its counterpart in *E. carotovora* subsp. *atroseptica*. These are members of a growing family of proteins related to Nep1 from *Fusarium oxysporum* which can induce necrotic responses in a variety of dicotyledonous plants. Nip_{Ecc} produced necrosis in tobacco, Nip_{Eca} affected potato stem rot, and both affected virulence in potato tubers. In *E. carotovora* subsp. *carotovora*, *nip* was shown to be subject to weak repression by the LuxR family regulator, EccR, and may be regulated by the negative global regulator RsmA.

Additional keywords: secretion, soft rot.

Quorum sensing is an important regulatory mechanism used to control a wide range of physiological processes in many bacterial species (Whitehead et al. 2001). In gram-negative bacteria, commonly used quorum-sensing systems rely upon a transcriptional regulator, similar to the LuxR protein of *Vibrio fischeri*, which is activated by a small signaling molecule, *N*-acyl homoserine lactone (or acyl HSL) (Eberhard et al. 1981). The former complexes with its cognate, acyl HSL, and regulates target gene expression by binding to specific sites in promoter regions. Expression of such genes generally is associated with high cell densities during the late exponential and early stationary phases of growth. Phenotypes regulated by acyl HSL-mediated quorum sensing include bioluminescence in *V. fischeri*, virulence factor production in *Pseudomonas aeruginosa*, and Ti plasmid conjugal transfer in *Agrobacterium tumefaciens* (Whitehead et al. 2001).

The phytopathogen *Erwinia carotovora* subsp. *carotovora* (Ecc) causes soft rot disease in many crops worldwide (Perembelton 2002). The closely related bacterium *E. carotovora* subsp. *atroseptica* (Eca) also causes soft rot disease but has a host range limited to potato in temperate regions. The genome sequence of *E. carotovora* subsp. *atroseptica* SCRI1043 has been completed recently (Bell et al. 2004).

The main determinant of soft rot in these pathogens is the production of an array of exoenzymes, including pectate lyases, polygalacturonases, cellulases, and proteases (Barras et al. 1994; Thomson et al. 1999). Some *E. carotovora* subsp. *carotovora* strains also produce the antibiotic, 1-carbapen-2-em-3-carboxylic acid (carbapenem) (Parker et al. 1982), although the *E. carotovora* subsp. *atroseptica* SCRI1043 does not make this molecule. Production of both carbapenem and exoenzymes is regulated by quorum sensing during the latter stages of growth in *E. carotovora* subsp. *carotovora* (Bainton et al. 1992; Jones et al. 1993). The system is dependent on the acyl HSL, *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL), which is synthesized by the *carI* (*expI* or *hslI*) gene product (Chatterjee et al. 1995; Jones et al. 1993; Pirhonen et al. 1993; Swift et al. 1993). In carbapenem regulation, OHHL interacts with a LuxR homologue, designated CarR (McGowan et al. 1995; Welch et al. 2000) which, once activated by the acyl HSL, induces the transcription of the carbapenem synthesis genes. For the regulation of exoenzymes, it is believed that OHHL interacts with another, as yet unidentified LuxR homologue that then controls the relevant target genes. A second LuxR homologue, EccR (ExpR or RexR) has been identified in *E. carotovora* subsp. *carotovora* strains (Andersson et al. 2000; Rivet 1998) and appears to be involved in the repression of exoenzyme synthesis (Andersson et al. 2000). However, this effect may be strain dependent (Rivet 1998).

When this study was initiated, little was known about the extent of quorum-sensing control within a single microorganism. Most research was directed toward demonstrating the existence of quorum-sensing systems within an organism or the characterization of a particular phenotype or phenotypes. Recently, however, genome-wide screens for quorum-sensing-controlled genes within some organisms have been performed using genome microarray and proteomic studies (Chen et al. 2003; Schuster et al. 2003; Wagner et al. 2003).

The aim of this study was to identify OHHL-regulated genes that encode proteins important in the soft rot *Erwinia* spp.–plant interaction. For this purpose, we used the *TnphoA* transposon (Manoil and Beckwith 1985) to mutagenize a phosphatase (*phoA*)-deficient *E. carotovora* subsp. *carotovora* ATTn10 derivative, because active *phoA* fusions are created only in genes encoding proteins exported to the periplasm, or beyond, thus targeting potential virulence-related proteins (Hinton and Salmond 1987). The expression of the reporter gene fusion then was assessed in the presence and absence of OHHL. In this way, OHHL-responsive fusions were isolated and seven novel quorum-sensing-dependent genes were identified.

RESULTS

Isolation of OHHL-responsive genes.

A Pho^- derivative of *E. carotovora* subsp. *carotovora* ATn10 (designated MS2) was isolated after chemical mutagenesis and screening of surviving colonies on the chromogenic substrate 5-bromo-4-chloro-3-indolyl-phosphate- p -toluidine (X-P). The MS2 mutant was prototrophic and not impaired in growth in Luria-Bertani broth (LB) (data not shown).

To enable screening for genes under quorum-sensing control, production of the signaling molecule OHHL first was abolished by transducing a *carI::TnblaM* mutation from strain JM17 (McGowan et al. 1995) into MS2 using the generalized transducing phage ϕ KP (Toth et al. 1993). The resulting strain was designated MS20. Disruption of *carI* and resulting elimination of OHHL production were confirmed using the acyl HSL detector strain *Chromobacterium violaceum* CV026 (McClellan et al. 1997) and assays for the quorum-sensing-dependent production of exoenzymes and carbapenem (data not shown).

To identify OHHL-regulated genes, *E. carotovora* subsp. *carotovora* MS20 (pHCP2) was infected with a high titer (1×10^{11} PFU ml^{-1}) lysate of λ TnphoA (Clement et al. 1982; Wilmes-Riesenberg and Wanner 1992) and transductant colonies selected on nutrient agar supplemented with kanamycin, OHHL, and X-P. Approximately 3% of transductants were

blue, indicating a Pho^+ phenotype. To differentiate between OHHL-responsive and nonresponsive gene fusions, blue colonies were rescreened on X-P in the presence and absence of OHHL and 43 of 3,626 (1.18%) displayed a positive response to the presence of OHHL.

To identify strains carrying TnphoA insertions in OHHL-repressible genes, MS20 was mutagenized again, with transductants selected on NA^{Km} and X-P without OHHL. Blue transductant colonies then were rescreened on NA^{Km} , OHHL, and X-P, resulting in 4 of 1,550 (0.26%) showing reduced expression of Pho in the presence of OHHL compared with those in the absence of OHHL (data not shown).

Identification and analysis of quorum-sensing-controlled genes.

Following transduction of each transposon insertion into a clean genetic background (MS20), the presence of a single insertion in each strain was confirmed using Southern blotting with a transposon-specific probe (data not shown). All fusion strains produced wild-type levels of exoenzymes and carbapenem (data not shown), suggesting that transposon insertions were not located in genes encoding the major exoenzymes or carbapenem but in novel, quorum-sensing-controlled genes. The absence of any such insertions in major exoenzyme or carbapenem synthesis genes indicates that the mutagenesis did not saturate the entire *E. carotovora* subsp. *carotovora* genome.

Table 1. The novel quorum-sensing genes of *Erwinia carotovora* subsp. *carotovora*^a

Gene	Effect of OHHL on expression	<i>E. carotovora</i> subsp. <i>atroseptica</i> protein similar to the predicted gene product	Reference and accession number for similar protein from database
<i>oig1</i>	15-Fold upregulation	ECA3946 (4.5e-177). Protein of unknown function.	No significant matches.
<i>oig2</i>	Fivefold upregulation	ECA3087 (8e-4). Similar to necrosis-inducing proteins, including Nep1 in <i>Fusarium oxysporum</i> .	AAC97382, Nelson et al. 1998
<i>oig3</i>	Twofold upregulation	ECA2220 (3.1e-15). Putative cellulase similar to <i>Bacillus subtilis</i> YoaJ protein.	AAB84448, Muller et al. 1992
<i>oig4</i>	Fourfold upregulation	No similar <i>E. carotovora</i> subsp. <i>atroseptica</i> protein. Similar to pectate lyase (PelL) from <i>E. chrysanthemi</i> .	AAF05308, Park et al. 2000
<i>org1</i>	Threefold downregulation	<i>E. carotovora</i> subsp. <i>atroseptica</i> pectate lyase (PelX) (4.8e-68) and PelX from <i>E. chrysanthemi</i> and <i>Klebsiella oxytoca</i> .	CAB39324, Shevchik et al. 1999
<i>org2</i>	2.5-Fold downregulation	ECA0053 (1.2e-19). Similar to <i>Vibrio vulnificus</i> 5'-nucleotidase/2'-3'-cyclic phosphodiesterase.	AAO11113, Kim et al. 2003
<i>org3</i>	Barely expressed, threefold upregulation in absence of OHHL	No similar <i>E. carotovora</i> subsp. <i>atroseptica</i> protein. Similar to putative haemagglutinin-related protein from <i>Ralstonia solanacearum</i> (2e-37).	CAD16950, Salanoubat et al. 2002

^a Novel quorum-sensing-regulated genes, together with a measurement of the induction or repression effect of the signaling molecule *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL). In each case, figures refer to the effect on gene expression during the early stationary phase of growth, compared to an MS2 wild-type strain. Proteins identified by tBLASTx database searches that share highest sequence similarity with each gene product are also shown, together with the *e* value for this similarity (in brackets).

Fig. 1. Quorum-sensing regulation of the novel genes in *Erwinia carotovora*. Alkaline phosphatase activity was measured in each *E. carotovora* subsp. *carotovora* mutant over 12 h of growth. Data represent phosphatase activity in a CarI^- strain (open bars), a CarI^+ strain (black bars), and in a CarI^- strain with exogenously added *N*-(3-oxohexanoyl)-L-homoserine lactone (striped bars) at $1 \mu\text{g ml}^{-1}$. Error bars represent $\pm$ one standard deviation from mean phosphatase activity. Data represent the average of three separate experiments.

To identify the location of each fusion, chromosomal DNA from each of the strains was digested with restriction enzymes to yield fragments of DNA containing the transposon insert. These fragments were ligated to pACYC184 and used to transform *Escherichia coli* DH5 α . The primer Tn5Pr1, which is complementary to the right and left IS50 elements of TnphoA, then was used to sequence across the fusion junction between chromosomal DNA and transposon. In combination with Southern hybridization analysis (using probes designed to generated sequence), this revealed that the 43 OHHL-inducible fusion strains had been created by insertions at different locations within four different genes. These were designated *oig1-4* for OHHL-inducible genes (Table 1). The four OHHL-repressible fusion strains were created by insertions in three different genes, and were designated *org1-3* for OHHL-repressible genes (Table 1).

The generated sequence then was used to search the recently completed *E. carotovora* subsp. *atroseptica* genome sequence for similar sequences (Table 1) (Bell et al. 2004). Where no similarity exists with *E. carotovora* subsp. *atroseptica*, translated sequences were used to screen the GenBank database using BLAST. The best of these hits are shown in Table 1 with a measure of this similarity.

Representatives of each of the seven classes of gene fusion were transduced into clean *E. carotovora* subsp. *carotovora* MS2 backgrounds using ϕ KP. The expression of each gene in *carI*⁺ (MS2) and *carI*⁻ (MS20) backgrounds, with and without the addition of exogenous OHHL, was analyzed using PhoA assays (Brickman and Beckwith 1975). OHHL-inducible fusions *oig1*, *oig2*, and *oig4* were found to be almost completely dependent on OHHL for their expression (Fig. 1; Table 1), mimicking the expression of genes encoding carbapenem and exoenzymes in *E. carotovora* subsp. *carotovora* (Bainton et al. 1992; Jones et al. 1993). The expression of *oig3*, however, was increased only by the presence of OHHL. The OHHL-repressible fusions *org1* and *org2* were downregulated by the presence of the OHHL, whereas expression of *org3* was tightly repressed. In all cases, the time at which gene expression was induced during growth was unaffected (data not shown).

The effect of mutation of each of these genes on the virulence of *E. carotovora* subsp. *carotovora* in potato tubers also was investigated using an inoculum size of 10⁶ cells. The amount of rotted tissue produced by each mutant was measured and the fusion strains with transposon insertions in the OHHL-inducible genes (with the exception of *oig2*, which is discussed further below) and *org2* were found to be as virulent as the wild type under the conditions investigated. However, roles for *org1* and *org3* in virulence in planta were indicated (Fig. 2).

Nip, a protein similar to a type of fungal elicitor.

The product of *oig2* shows similarity to a growing family of proteins related to Nep1, the elicitor of plant necrosis from *Fusarium oxysporum* (Bailey 1995; Pemberton and Salmond 2004). These have been identified in both phytopathogenic and saprophytic bacteria, oomycetes, and fungi (Fig. 3). Little is currently known about the function of these proteins or the reason for their conservation in such diverse organisms. However, nearly all members of the family, designated Nep1-like proteins (NLPs), contain conserved residues, including two cysteine residues and a central GHRHDWE motif. These all were found in Oig2. Based on its similarity to the NLPs, Oig2 was designated an *E. carotovora* subsp. *carotovora* necrosis-inducing protein (Nip_{Ecc}). A protein with 81% identity to Nip_{Ecc} also was identified in *E. carotovora* subsp. *atroseptica* SCRI1043 (Bell et al. 2004), and designated Nip_{Eca}. The *nip* locus from this organism is shown in Figure 4A. Although *nip* could be formally operonic with the genes encoding proteins

of unknown function that are found upstream from its start codon, it would form the last gene of this operon. Therefore, any mutation of *nip*_{Eca} will not have polar effects on the transcription of downstream genes.

With the possible exception of the homologue in *Neurospora crassa*, all NLPs are either secreted or predicted to be extracellular. The presence of an N-terminal signal sequence and its cleavage site in Nip_{Ecc} was confirmed by expression of the protein from the plasmid pNIP1 in *Escherichia coli* M15 pREP4. This plasmid was constructed by inserting *Erwinia carotovora* subsp. *carotovora* *nip* with its native ribosome-binding site into the

Fig. 2. Potato tuber virulence assay for the *Erwinia carotovora* subsp. *carotovora* *org* mutants. Quorum-sensing mutants containing insertions in genes **A**, *org1*, **B**, *org2*, and **C**, *org3* were compared with a wild-type strain (MS2) for the ability to rot potato tubers. An inoculum size of 10⁶ cells was used in each case. Assays also were performed for *oig1*, *oig3*, and *oig4*. The profiles generated by these mutants resembled that of *org2*. Error bars represent $\pm$ one standard deviation from the mean rot produced (in grams) from six potatoes.

vector pQE30 without the inclusion of the vector 6-His tag. Using N-terminal sequencing, Nip_{Ecc} isolated from the periplasmic fraction of *Escherichia coli* M15 pREP4 pNIP1 was found to have lost a 22-amino-acid signal sequence (Fig. 3).

Nip_{Ecc} induces cell death in planta.

Other members of the NLP family, including those in *F. oxysporum*, *Pythium aphanidermatum*, and *Phytophthora sojae*, induce necrotic responses in a variety of dicotyledonous plants

Fig. 3. Alignment of the Nip1-like proteins (NLPs). Shading indicates sequence conservation with 100% (black), 80 to 99% (dark gray), and 60 to 79% (light gray) conservation shown. The sequences featured are from *Erwinia carotovora* subsp. *carotovora* (Ecc), *E. carotovora* subsp. *atroseptica* (Eca), *Fusarium oxysporum* (Fo), *Pythium aphanidermatum* (Pa), *Phytophthora infestans* (Pi), *P. parasitica* (Pa), *P. sojae* (Ps), *Neurospora crassa* (Nc), *Vibrio pommerensis* sp. CH-291 (Vp), *Bacillus halodurans* (Bh), and *Streptomyces coelicolor* (Sc). The signal sequence of Nip_{Ecc} is underlined.

Fig. 4. **A**, The *nip* locus of *Erwinia carotovora* subsp. *atroseptica* SCRI1043. The *nip* gene is shown (in white) together with the genes surrounding it (in gray). Genes are labelled with ECA gene numbers and predicted products as designated in the *E. carotovora* subsp. *atroseptica* SCRI1043 genome sequence (Bell et al. 2004). **B**, Production of necrosis in tobacco by heterologously-expressed Nip_{Ecc}. Cell death in tobacco leaves induced by Nip_{Ecc}. Panels were infiltrated with control strain *Escherichia coli* M15 pREP4 pQE30 or *E. coli* M15 pREP4 pNIP1 as shown. Infiltrations were performed with sonicated cultures containing approximately 1×10^9 CFU ml⁻¹. Picture taken after 24 h of incubation at 25°C. The scale bar shown represents size in millimeters. This experiment was repeated on groups of six to eight plants on at least four separate occasions with the same result.

(Bailey 1995; Koch et al. 1998; Qutob et al. 2002). To test whether this was also true of Nip_{Ecc}, *E. coli* M15 pREP4 cultures containing pNIP1 or pQE30 (vector without *nip* insertion) were suspended in 10 mM magnesium chloride, standardized to a concentration of 10⁹ CFU ml⁻¹, sonicated, and then infiltrated into opposing panels of *Nicotiana tabacum* L. cv. Xanthi-nc leaves. In all leaves, cell death was observed only in panels infiltrated with sonicate from cells expressing Nip_{Ecc} (Fig. 4B). This cell death, initially visible as localized necrotic lesions, was seen at 9 to 10 h following infiltration. Within 48 h, the lesions had merged and tissue in the infiltrated leaf panel had collapsed. No response was observed in leaf sections infiltrated with sonicates of cells containing the vector only or the resuspension solution (10 mM magnesium chloride) controls.

To determine whether the site of Nip_{Ecc} application affects the production of leaf necrosis, spots of M15 pREP4 pNIP1 were applied to the adaxial surface of the leaf. No cell death was observed, suggesting that Nip_{Ecc} is unable to cross the leaf cuticle and requires infiltration into the leaf to cause a response.

Fig. 5. Potato stem pathogenicity assay of *Erwinia carotovora* subsp. *atroseptica* (Eca). Pathogenicity assay showing **A**, wild-type *E. carotovora* subsp. *atroseptica* SCRI1043 and **B**, PA1420 (*nip::Tn5Km^r*) on potato (cv. Estima) stems following inoculation with 10⁶ cells/inoculation site and incubation at 22°C for nine days. **C** Comparison of lesion development between *E. carotovora* subsp. *atroseptica* SCRI 1043 wild type and PA1420 at two inoculation concentrations (10² and 10⁶ cells/inoculation site). Least significant difference values were 1.6 and 12.6 for the low and high inocula, respectively ($P < 0.05$). Data presented shows average length of 12 replicates.

Nip_{Eca} functions in the development of stem rot in potato.

To obtain a *nip* mutant of *Erwinia carotovora* subsp. *atroseptica* SCRI1043, a pool of mutants generated by random mutagenesis using the transposon mTn5-*gusA*-pgfp21 (Xi et al. 1999) was screened using polymerase chain reaction (PCR). Once identified, the in vitro growth rates of PA1420 (*nip::Tn5Km^r*) and the wild-type strain in LB were compared and found to be similar (data not shown). Potato plant stems then were inoculated with either 10⁶ or 10² PA1420 CFU per inoculation site and compared with the equivalent level of the wild-type strain over nine days (Fig. 5). At both inoculum concentrations, lesion length was significantly reduced ($P < 0.05$) in stems inoculated with the mutant compared with the wild-type strain over the same period, with reductions of approximately 62 and 39% at inoculum concentrations of *E. carotovora* subsp. *atroseptica* at 10⁶ and 10² CFU/inoculation site, respectively (Fig. 5). Multiple attempts were made to genetically complement the reduced-virulence phenotype using the *nip* gene cloned in trans, on various plasmids of different copy number. However, all derivatives of both the Nip mutant and the wild-type *E. carotovora* subsp. *atroseptica* host carrying the plasmid vectors, with or without the *nip* gene, showed seriously diminished virulence in potato stem assays, presumably because of a pronounced fitness cost of plasmid maintenance in the potato stem environment (data not shown). Thus, it proved impossible to assess complementation effects in these stem inoculation assays.

Nip_{Ecc} is involved in soft rot production in potato tubers.

To determine whether Nip_{Ecc} or Nip_{Eca} is involved in the development of soft rot in potato, tuber assays were performed to

Fig. 6. Potato tuber assay for *Erwinia carotovora* subsp. *carotovora* (Ecc) and *E. carotovora* subsp. *atroseptica* (Eca) *nip* mutant strains. Potato (cv. Estima) tuber assays were performed for both wild-type and *nip* disruption strains of *Erwinia carotovora* subsp. *carotovora* and *E. carotovora* subsp. *atroseptica* using an inoculation size of 10⁵ cells. **A**, *Erwinia carotovora* subsp. *carotovora* MS2 (wild type) and CP1000 (*nip::TnphoA*) are compared. **B**, *E. carotovora* subsp. *atroseptica* SCRI1043 (wild type) and *E. carotovora* subsp. *atroseptica* PA1420 (*nip::Tn5Km^r*) mutant. Error bars represent $\pm$ one standard error from the mean rotten tissue obtained from 16 potatoes in two separate experiments.

compare the levels of tissue rot between the *E. carotovora* subsp. *carotovora* mutant CP1000 (*nip::TnpH*A) and MS2 (wild type), and between the *E. carotovora* subsp. *atroseptica* mutant PA1420 (*nip::Tn5Km*^r) and SCRI1043 (wild type). All LB-only controls were negative for soft rot.

At an inoculum density of 10⁵ CFU/inoculation site, there was no observable difference between the *nip* mutants and wild-type strains (data not shown). However, at 10² CFU/inoculation site, more tissue rot was generated by the wild-type strains than by their respective *nip*-mutants (Fig. 6). Student's *t* test showed a statistically significant difference (*P* < 0.05) between the *E. carotovora* subsp. *carotovora* mutant and its wild-type strain at 72 and 96 h. At each time point during the tuber assays, viable cell counts were performed but no differences between the numbers of wild-type or *nip* mutant cells were observed with either *E. carotovora* subsp. *carotovora* or *atroseptica* (data not shown). More importantly, unlike the problematic attempts at genetic complementation assays with the stem inoculation tests, provision of the wild-type *nip* allele in trans fully restored the *Nip* mutant to wild-type levels of rotting in tuber assays (Fig. 7).

The regulation of *nip* expression in *E. carotovora* subsp. *carotovora*.

To investigate the role of quorum sensing in *nip* expression, the impact of the two LuxR-like proteins in *E. carotovora* subsp. *carotovora*, EccR and CarR was investigated using the *E. carotovora* subsp. *carotovora* mutant CP1000 (*nip::TnpH*A). Mutations *eccR::TnpH*A-2 from *E. carotovora* subsp. *carotovora* A14 (S. Harris, unpublished data) and *carR::TnpH*A-2 from *E. carotovora* subsp. *carotovora* GB3 (Bosgelmez 1999) were transduced into CP1000 and CP1016 (*nip::TnpH*A and *carI::TnblaM*) backgrounds using ϕ KP. The levels of *nip* transcription then were monitored throughout growth using PhoA assays. The *carR* mutation had no impact on *nip* expression (data not shown). However, the disruption of *eccR* led to a slight increase (approximately 1.2-fold during early stationary phase) in *nip* transcription in the CP1000 background (Fig. 8A). In CP1024 (*nip::TnpH*A, *carI::TnblaM*, and *eccR::TnpH*A-2), the *eccR* mutation was found to have no impact on *nip* expression.

A link between the RsmA/*rsmB* regulatory network and quorum-sensing control of genes encoding exoenzymes in *E. carotovora* subsp. *carotovora* has been reported previously (Chatterjee et al. 2002; Koiv and Mae 2001). To determine whether this network has any effect on *nip* expression, *rsmB::TnpH*A-2 derivatives of CP1000 and CP1016 were created by ϕ KP-mediated transduction from *E. carotovora* subsp.

Fig. 7. Allelic complementation assay with the *Erwinia carotovora* subsp. *atroseptica* *Nip* mutant. The tuber rotting assay was performed after 96 h, comparing the wild-type *E. carotovora* subsp. *atroseptica* SCRI 1043 carrying the vector plasmid pCL1920; PA1420 carrying pCL1920; and PA1420 carrying the plasmid with the wild-type *nip* allele in trans, pNIP20.

carotovora C36 (S. Harris, unpublished data). By following *nip* expression throughout growth in Pho assays, a role for the *rsm* system in *nip* regulation was demonstrated. The mutation of *rsmB*, in both CarI⁺ and CarI⁻ strains, was found to reduce *nip* levels to those observed in CP1016 (Fig. 8B). The observed *nip* expression levels were reduced four- to fivefold compared with the wild-type during early stationary phase.

Mutation of *rsmB*, the antagonist of RsmA function, is likely to result in an increase in levels of the functional regulator. To determine whether the downregulation of *nip* expression in an *rsmB* mutant was due to repression, either directly or indirectly, by RsmA, strain CP1000 was transformed with plasmids pSH58 (*E. carotovora* subsp. *carotovora* *rsmA* in pCL1920) and pCL1920. The expression of *nip* throughout growth then was followed using PhoA activity (Fig. 8B). The possession of pCL1920 alone was found to cause a slight reduction in expression levels. However, possession of pSH58 resulted in a reduction to levels seen in an *rsmB* mutant. These data suggest that RsmA may have a repressive effect on *Nip* production in *E. carotovora* subsp. *carotovora*.

DISCUSSION

The aim of this study was to identify novel quorum-sensing-controlled proteins with a possible role in the soft rot *Erwinia*

Fig. 8. The regulation of *nip* transcription in *Erwinia carotovora* subsp. *carotovora*. Alkaline phosphatase data showing levels of *nip::TnpH*A expression in **A**, CP1000 (*nip::TnpH*A; all further mutants constructed in this genetic background), CP1016 (*carI::TnblaM*), CP1022 (*eccR::TnpH*A-2), and CP1024 (*eccR::TnpH*A-2, *carI::TnblaM*); and **B**, CP1000, CP1016, CP1027 (*rsmB::TnpH*A-2), and CP1028 (*rsmB::TnpH*A-2, *carI::TnblaM*). Strains CP1000 pSH58 (*E. carotovora* subsp. *carotovora* *rsmA* in pCL1920) and CP1000 pCL1920 also are shown. Broken lines indicate bacterial growth while unbroken lines show alkaline phosphatase activity. Error bars represent $\pm$ one standard deviation from the mean phosphatase activity. Data represents the average of three separate experiments.

spp.–plant interaction. *TnphoA* was used as the mutagen to enrich for mutants affected in possible periplasmic, transmembrane, outer membrane, and secreted proteins, while omitting cytoplasmic protein mutants. As a result, a much smaller subset of the total number of quorum-sensing-regulated genes in *E. carotovora* subsp. *carotovora* was identified compared with, for example, recent microarray analysis in *Pseudomonas aeruginosa*, where approximately 6% of the genome was shown to be under quorum-sensing control (Schuster et al. 2003). Of the quorum-sensing-controlled genes identified, however, a higher proportion of those with exported products were quorum-sensing induced rather than repressed.

Identification and possible functions of genes controlled by quorum sensing.

We identified seven novel quorum-sensing-regulated genes. Four were induced (designated *oig1* to 4) and three were repressed (*org1* to 3) by the signaling molecule OHHL, highlighting the complexity of the quorum-sensing system in *E. carotovora* subsp. *carotovora*. Three of the OHHL-induced genes were found to be almost completely dependent on the signaling molecule for their expression, as are genes that encode carbapenem and at least some of those encoding exoenzymes (Bainton et al. 1992; Jones et al. 1993). Expression of *oig3*, however, was increased by OHHL but not entirely dependent on it. Similar differences were seen in the OHHL-repressed genes, where the signal molecule tightly repressed *org3* expression but only reduced expression of *org1* and *org2*.

Oig3 shares sequence similarity with the YoaJ protein, from *Bacillus subtilis*, which resembles PapC from *Escherichia coli* (Muller et al. 1992). The latter is a membrane usher protein and part of the system responsible for folding and assembly of the P pilus in the outer membrane of uropathogenic *E. coli* (Thanassi et al. 1998). Like its putative *E. coli* counterpart, *Oig3* is predicted to be periplasmic (PSORT prediction). Gene *oig3* expression was upregulated by quorum sensing at low cell density, indicating that its protein may be important at low levels during noninfective growth as well as during infection (Fig. 1). This expression pattern is not surprising if, like PapC, *Oig3* is involved in host cell attachment. Disruption of *oig3* had no obvious effect on virulence in potato tubers, although it is possible that this crude assay circumvents the need for the *Oig3* protein.

The identity of *Oig1* is unclear. PSORT predictions indicate that the protein is likely to be localized to the outer membrane. Gene *oig1* in *Erwinia carotovora* subsp. *atroseptica* has a lower average G+C content (44%) than the rest of the genome (50.9%) (Bell et al. 2004), suggesting possible acquisition from another organism by horizontal transfer.

Oig4 and *Org1* show similarity to minor pectate lyases (PelL and PelX, respectively) from *E. chrysanthemi* (Park et al. 2000; Shevchik et al. 1999) and to proteins from *E. carotovora* subsp. *atroseptica*. Enzyme phenotypes were not apparent in such mutants during exoenzyme screens on agar plates, consistent with the absence of any visible reduction in virulence on potato tubers in *oig4*-disrupt strains (data not shown). The dependence of *oig4* on quorum sensing for transcription suggests that it may be required during a specific stage of infection. The different Pel isoenzymes in *E. chrysanthemi* are thought to be sequentially expressed, possibly in response to changes in environmental pH (Nachin and Barras 2000). *Oig4*, like PelL of *E. chrysanthemi*, may have relatively low activity on polygalacturonate but may still play an important role at a specific stage of infection (Roy et al. 1999). The repression of *org1* at high cell densities does not resemble the behavior of its *E. chrysanthemi* counterpart, *pelX*. This is induced by oxygen limitation and pectate catabolism products (Shevchik et al.

1999) which are more likely at high cell density and, therefore, high OHHL concentrations. Thus, *Org1* in *E. carotovora* subsp. *carotovora* may have a role early in infection.

The gene product of *org2* is similar to a nucleosidase from *Vibrio vulnificus* (Kim et al. 2003) and may play a role in nutrient scavenging. This would explain its repression during infection, where nutrients are more readily available, and also why its disruption has no obvious effect on virulence. *Org3* shows sequence similarity to a putative haemagglutinin from *Ralstonia solanacearum* (Salanoubat et al. 2002). Its expression was completely repressed by quorum sensing, which is unusual because haemagglutinins are often important virulence factors. However, it may still have a role in virulence, possibly during initial attachment.

Biological properties

of Nip, a homologue of a fungal elicitor of necrosis.

Oig2 (Nip_{Ecc}) shows similarity to a family of proteins from fungi and bacteria, some of which induce plant cell necrosis (Bailey 1995; Fellbrich et al. 2002; Koch et al. 1998). Based on similarity searches, a gene was identified in the *E. carotovora* subsp. *atroseptica* SCRI1043 genome, the product of which shares 81% identity with Nip_{Ecc}.

Although no precise biochemical function has yet been assigned to the NLP family, several members produce necrotic cell death in dicotyledons (Bailey 1995; Fellbrich et al. 2002; Koch et al. 1998). When expressed in *Escherichia coli*, Nip_{Ecc} produced necrotic cell death in tobacco that resembled the hypersensitive response (HR), where programmed cell death occurs at the site of pathogen infection. However, it is not clear whether the necrosis observed with Nip_{Ecc} is a result of an HR-like response or a direct killing effect on plant cells, although data from other NLP responses suggests that it may be HR-type cell death (Fellbrich et al. 2002; Koch et al. 1998).

Despite producing plant cell necrosis, there is little data to suggest that the NLPs are vital for the production of plant disease. For example, the disruption of *Nep1* has no effect on the pathogenicity of *F. oxysporum* on *Erythroxylum coca* (Bailey et al. 2002). The role of Nip_{Ecc} in *Erwinia carotovora* subsp. *carotovora*-produced tobacco leaf necrosis is not clear because the production of soft rot by the bacterium masks any necrotic effect. Spot inoculations to compare soft rot of a wild-type and *nip* mutant in tobacco did not demonstrate any clear difference (data not shown). therefore, the precise role for the protein in soft rot production by *E. carotovora* subsp. *carotovora* in tobacco is unclear.

Nip_{Ecc} and Nip_{Eca} do, however, appear to function in the pathogenesis of *Erwinia* spp. on potato. A clear reduction in soft rot on potato tubers was observed in the later stages of infection (72 and 96 h postinfection) of an *E. carotovora* subsp. *carotovora nip* mutant. This effect was less obvious in *E. carotovora* subsp. *atroseptica*. In potato stem inoculations, a role for Nip_{Eca} was observed. *E. carotovora* subsp. *atroseptica nip* mutants showed a 62% reduction in rotting compared with the wild-type strain at an inoculation level of 10⁶ cells, although the reason for this reduction in rotting is unclear. Both *E. carotovora* subsp. *carotovora* and *atroseptica nip* mutants show no alteration in exoenzyme production levels. It may be that the interaction between *nip* and plant cells promotes virulence either directly or via a protective function, possibly by interacting with components of the plant defense system. The latter hypothesis, however, seems unlikely because there was no obvious reduction in the survival of *nip* mutants from viable counts performed during potato tuber assays. A direct role in virulence seems more likely because, for example, the NLP homologue from *Vibrio pommerensis* CH-291 possesses some hemolytic activity (Jores et al.

2003). However, the function of NLPs as virulence factors raises questions over their presence in apparently saprophytic organisms like *Bacillus halodurans*, *Streptomyces coelicolor*, and *Neurospora crassa*. Nip_{Ecc} applied to external surfaces of tobacco leaves did not produce necrosis, suggesting that it is not able to cross the leaf cuticle and may thus require access to leaf intercellular spaces. This reflects the lifestyle of *Erwinia* spp., which only produce disease when inside plants. Bailey and associates (2002) observed that Nep1 from *F. oxysporum* applied as a foliar spray had the greatest effect at midday and after a long dew period, when stomata were open and access to the interior of the leaf was optimal.

The presence of a classical secretion signal in Nip_{Ecc} was demonstrated using N-terminal sequencing of protein samples obtained from the periplasm of *Escherichia coli* strains expressing the protein, consistent with Nip_{Ecc} being transported across the inner membrane of *Erwinia carotovora* subsp. *carotovora* using the Sec system. The secretion system used to traverse the outer membrane is not yet known.

Regulation of *nip* expression in soft rot *Erwinia* spp.

In *E. carotovora* subsp. *carotovora*, *nip* is under quorum-sensing control, although the mechanism of this control is unclear. A fivefold reduction in *nip* levels was observed upon disruption of *carI*, the gene encoding OHHL synthase. However, because neither CarR nor EccR was responsible for regulation to this level (Fig. 8A), the chain of command between OHHL production and *nip* regulation in *E. carotovora* subsp. *carotovora*, as for exoenzyme genes, remains elusive.

To identify regulators that may play a role in *nip* regulation by quorum sensing, the effect of the Rsm system was investigated. This consists of the RNA-binding negative regulator RsmA and the untranslated *rsmB* mRNA, which is believed to act as an antagonist of the protein, using sequestration to neutralize its negative effects (Cui et al. 1995; Liu et al. 1998). It has been suggested that the effects of OHHL in *E. carotovora* subsp. *carotovora* may be achieved through the Rsm system (Chatterjee et al. 2002; Koiv and Mae 2001). RsmA previously has been described as a repressor of genes in the interaction between an *Erwinia* sp. and its plant hosts (Mukherjee et al.

1996). RsmA also reduces the ability of *E. amylovora* to elicit a hypersensitive response (Mukherjee et al. 1996).

We determined the role of *rsmB* in *nip* expression by measuring the activity of *nip::TnphoA* in an *rsmB*⁻ mutant with either CarI⁺ or CarI⁻ backgrounds. In both strains, *nip* levels were the same as those observed in a *carI*-only disruption strain (approximately 4 to 5× reduction in expression compared with the wild type). The provision of *rsmA* in trans produced a similar effect.

Thus, RsmA may repress *nip* expression, concurring with the proposed model in which the absence of OHHL results in increased levels of RsmA by an unknown mechanism (Chatterjee et al. 2002; Koiv and Mae 2001). This suggests that in a CarI⁻ background, RsmA levels are greatly increased while *nip* levels are reduced, as seen with exoenzyme levels in *E. carotovora* subsp. *carotovora* (Chatterjee et al. 2002). The same pattern is seen in an *rsmB* disruption strain where there is no antagonism of RsmA activity. The similar levels in the *rsmB*⁻ *carI*⁻ mutant may suggest that RsmA levels have reached a maximum and no further repression of *nip* is possible. These data agree with work by Mattinen and associates (M. Pirhonen, *personal communication*), who observed an increased production of Nip in *E. carotovora* subsp. *carotovora* SCC 3193 RpoS mutants which are known to have reduced RsmA levels. It is, however, unclear whether RsmA has a direct effect on *nip* transcript stability or whether this repression occurs through an intermediate step, such as the regulation of another transcriptional regulator.

In conclusion, we have successfully identified seven novel genes involved in quorum sensing in *E. carotovora* subsp. *carotovora*, and showed that at least one of these, *nip*, is involved in pathogenesis on potato and can induce necrosis in tobacco. Further work is required to understand their regulation and potential roles in the pathogenicity of this successful plant pathogen.

MATERIALS AND METHODS

Strains and media.

Strains used during this study are listed in Table 2. Unless stated otherwise, all cultures were grown in LB, M9 minimal medium (Sambrook et al. 1989), or on nutrient agar plates at 25 (*E. carotovora* subsp. *atroseptica*), 30 (*E. carotovora* subsp. *ca-*

Table 2. Bacterial strains used in this investigation

Strain	Genotype, characteristics ^a	Reference
<i>Escherichia coli</i>		
DH5α	...	Hanahan, 1983
M15(pREP4)	Protein expression strain	Qiagen, Crawley, UK
ESS	β-lactam-supersensitive indicator strain	Beecham Pharmaceuticals
S17-1λpir (mTn5-gus-pgfp21)	S17-1λpir carrying the mTn5-gus-pgfp21 cassette ("Tn5 cassette") in pUT, Km ^r , Ap ^r	Xi et al. 1999
<i>Erwinia carotovora</i> subsp. <i>carotovora</i>		
ATTn10	Wild type	McGowan et al. 1996
JMJ7	<i>carI::TnblaM</i> derivative of ATTn10	McGowan et al. 1995
MS2	EMS-induced phosphatase-negative mutant of ATTn10	This work
MS20	<i>carI::TnblaM</i> derivative of MS2	This work
A14	<i>eccR::TnphoA</i> -2 derivative of ATTn10	S. Harris, personal communication
C36	<i>rsmB::TnphoA</i> -2 derivative of ATTn10	S. Harris, personal communication
GB3	<i>carR::TnphoA</i> -2 derivative of ATTn10	Bosgelmez, 1999
CP1000	<i>nip::TnphoA</i> derivative of MS2	This work
CP1016	<i>nip::TnphoA</i> derivative of MS20	This work
CP1022	<i>eccR::TnphoA</i> -2 derivative of CP1000	This work
CP1024	<i>eccR::TnphoA</i> -2 derivative of CP1016	This work
CP1027	<i>rsmB::TnphoA</i> -2 derivative of CP1000	This work
CP1028	<i>rsmB::TnphoA</i> -2 derivative of CP1016	This work
<i>E. carotovora</i> subsp. <i>atroseptica</i>		
SCRI 1043	Wild type	SCRI bacterial collection
PA1420	SCRI1043 with insertion of the Tn5 cassette in <i>nip</i> (<i>nip::Tn5Km^r</i>)	This work
<i>Chromobacterium violaceum</i>		
CV026	OHHL production indicator strain	McClean et al. 1997

^a Km = , Ap = ampicillin, and OHHL = *N*-(3-oxohexanoyl)-L-homoserine lactone.

rotovora and *C. violaceum*), and 37°C (*Escherichia coli*). Where required, X-P and antibiotics were added to a final concentration of 50 µg ml⁻¹ unless stated otherwise. For *E. coli* M15 pREP4 cultures, the pREP4 plasmid was maintained by addition of Km at 25 µg ml⁻¹. Exogenous OHHL (Paul Williams, Nottingham University, U.K.) was added to a final concentration of 1 µg ml⁻¹. During cloning steps, plasmids pACYC184 (New England Biolabs Ltd., Hitchin, U.K.) and pQE30 (Qiagen Ltd., Crawley, U.K.) were used as described. Plasmid pSH58, containing *Erwinia carotovora* subsp. *carotovora* *rsmA* in pCL1920, was made by S. Harris (*unpublished data*).

Chemical mutagenesis.

Chemical mutagenesis of *E. carotovora* subsp. *carotovora* ATTN10 was carried out using ethylmethane sulphonate (EMS) as described previously (Bainton et al. 1992). Colonies recovered after mutagenesis were analyzed for phosphatase activity on nutrient agar plates containing X-P. All phosphatase-negative candidates were analyzed for possible auxotrophy and their growth rates compared with that of the progenitor wild-type strain.

Transposon mutagenesis and generalized transduction.

Transposon mutagenesis of *E. carotovora* subsp. *carotovora* MS20 using λTnphoA (Wilmes-Riesenberg and Wanner 1992) was performed as described by Harris and associates (1998). Generalized transduction of *E. carotovora* subsp. *carotovora* and *atroseptica* strains using φKP and φM1, respectively, was as described by Toth and associates (1993, 1997).

Exoenzyme plate assays.

Pectate lyase and cellulase activities were detected using plate assays as described by Andro and associates (1984). Protease activity was determined using the plate assay of Hankin and Anagnostakis (1975). Samples (5 µl) of an overnight culture of the microorganism to be tested were spotted onto the appropriate enzyme assay plate and incubated at 30°C for 36 h.

Carbapenem plate assay.

Strains to be tested for carbapenem production were grown overnight in LB at 30°C. Culture aliquots of 5 µl were spotted onto a lawn of *Escherichia coli* ESS on a nutrient agar plate and incubated at 30°C for 36 h before scoring inhibition halos due to the antibiotic.

DNA manipulation and sequence analysis.

Unless stated otherwise, all molecular biological techniques were performed according to Sambrook and associates (1989). Electroporations were performed as described by Dower and associates (1988) using a Bio-Rad Gene Pulser. DNA sequencing was carried out by Department of Biochemistry Sequencing Facility, Cambridge University. The primer Tn5PR1 (CAGGA CGTACTTGTG) was used to sequence across the transposon-gene-fusion junctions. Nucleotide sequence data were analyzed using the Genetics Computer Group software package (version 10.2 (Genetics Computer Group, Madison, WI, U.S.A.)). The deduced primary protein sequence was compared with sequences within the GenBank/EMBL and SWISS-PROT databases using BLAST.

PCR amplification was performed using standard protocols, with primers NIP1 (CGGAATTCATTGTAAGTCAAGG) and NIP10 (CGGAATTCGCTGTGTCGCCGCTTC) used to amplify the *oig2* (*nip*) gene from chromosomal DNA.

Alkaline phosphatase assay.

Samples were collected hourly throughout growth and sonicated using a Misonix sonicator before performing alkaline

phosphatase assays as described by Brickman and Beckwith (1975). Optical density readings at 420 nm (OD₄₂₀) were measured using a Helios λ Unicam spectrophotometer, and readings expressed as alkaline phosphatase activity per milliliter of sample per minute of incubation time (at 37°C) per optical density of the bacterial culture.

N-terminal sequencing.

E. coli M15 pREP4 pNIP1 cultures were grown as described and separated into cytoplasmic, periplasmic, and supernatant fractions as described by Neu and Heppel (1965). Proteins from these fractions then were separated on a 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and transferred to Immobilon-P polyvinylidene difluoride membrane (Millipore Corporation, Bedford, MA, U.S.A.) using electroblotting (Sambrook et al. 1989). The band corresponding to Nip_{Ecc} from the periplasmic fraction then was sequenced at the Protein and Nucleic Acid Facility, Cambridge University.

Transposon mutagenesis of *Erwinia carotovora* subsp. *atroseptica* SCRI1043 and PCR screening.

Escherichia coli S17-1 λpir, carrying the Tn5 cassette (mTn5-*gusA-pgfp21*) (Xi et al. 1999) in the pUT vector (donor strain), and *Erwinia carotovora* subsp. *atroseptica* SCRI1043 (recipient strain) were grown to exponential phase in LB, with Km in the case of *Escherichia coli*. Donor and recipient strains were conjugated in a 2:1 ratio of donor to recipient for 6 h at 27°C. Plates were washed with morpholineethanesulfonic acid (MES), and transconjugants were recovered on M9Km at 27°C. In all, 4,608 transconjugants were selected and grown in 12 384-well microtiter plates (M9Km broth plus 4% glycerol). All were tested for Km resistance and sensitivity to ampicillin (Ap) to confirm insertion of the transposon and loss of pUT. Cells from each microtiter plate were transferred to M9Km agar and, following incubation at 27°C for 48 h, DNA was extracted from each group of clones to give 12 pools of DNA for PCR screening.

Tn5 can insert in both orientations in the genome; therefore, each position of interest was searched with one of two possible "search primers" (both orientations) outside the transposon but in an area close to the gene of interest, together with the "Tn5 primer" designed to anneal to one border of the Tn5 cassette. Initially, all 12 plate pools were screened and, where a PCR product of the expected size was generated, individual wells from that plate were rescreened to identify the mutant clone. PCR products were purified using the Wizard PCR Preps DNA Purification System (Promega Corp., Madison, WI, U.S.A.) and sequenced in both directions using the ABI PRISM BigDye terminator cycle sequencing ready reaction kit (v2.0; PE Biosystems, Foster City, CA, U.S.A.) to confirm the location of the transposon.

Leaf infiltration assay.

The 50-ml cultures of *E. coli* M15 pREP4 carrying pNIP1 or pQE30 (control) were grown at 37°C in LB (supplemented with Ap and Km) until an OD₆₀₀ of 0.5 was achieved. At this point, 1 mM isopropylthio-β-D-galactoside was added and the culture was incubated at 37°C for a further 2 h. The culture then was centrifuged and the supernatant removed. The cellular pellet was resuspended in 50 ml of 10 mM magnesium chloride and sonicated until all cells had been disrupted.

Sonicated samples were injected into the leaf apoplast of 6- to 10-week-old tobacco plants (*Nicotiana tabacum* L cv. Xanthi-nc) using a 10-ml syringe applied to the lower surface of the leaf as described in Klement (1963), except that no needle was used. The progress of infiltration was monitored visually and injected leaf panels became translucent when air in the leaf

spaces had been replaced completely with the solution. After approximately 30 min, leaves regained their normal appearance due to the solution being taken up by the leaf cells. The infiltrated tobacco plants were maintained in a greenhouse at 25°C following injection and leaves were harvested at specified times after infiltration to monitor necrotic effects.

Tuber virulence assay.

The virulence of *Erwinia carotovora* subsp. *carotovora* strains was determined by local inoculation of intact Estima potato tubers as described by Walker and associates (1994). Starting inocula were 10⁵ or 10² cells/inoculation site. Replica tubers were harvested at 24 h time points over a 96-h period and the level of potato tissue maceration determined by cutting the tubers in half and removing and weighing the necrotic tissue. Control experiments in which LB was injected into a potato also were carried out. Complementation tests on the Nip mutant of *E. carotovora* subsp. *atroseptica* using the wild-type *nip* allele with native promoter cloned in trans into plasmid pCL1920 were done as above and scored for rot after 96 h.

Viable counts were performed using 0.1-g samples of rotted potato tissue resuspended in 1 ml of LB. Serial dilutions of this suspension then were performed using LB, spread onto LB agar plates, and incubated at the temperatures specified above.

Potato stem virulence assay.

Microplants of the potato cv. Estima (*E. carotovora* subsp. *atroseptica* susceptible) were obtained from a commercial source (Gentech, Dundee, U.K.), replanted in 15-cm pots containing compost, and grown to approximately 20 cm in height in a controlled glasshouse environment of 22°C with a 16-h light regime. Bacterial strains were grown to log phase (overnight) in LB broth at 27°C and cells were harvested by centrifugation at 4,000 × g for 10 min, washed in phosphate-buffered saline (PBS) (pH 7.4), and resuspended in the same volume of PBS. Two dilutions (10⁸ and 10⁴ cells ml⁻¹) were prepared in PBS from these suspensions, and potato plant stems were inoculated (under the second fully expanded leaf) with 10 µl of bacterial suspension (equivalent to 10⁶ and 10² cells/inoculation site) using a micropipette tip. Inoculation sites then were covered with parafilm to prevent desiccation. At least 12 replicate plants were used for each strain and at each inoculum. The inoculated plants were kept at 22°C with a 16-h light regime and high humidity, and scored daily over a 9-day period for symptom development, which was recorded as length of rot (mm). Genstat for Windows (version 6.1.0.200; Genstat 6th edition, Lawes Agricultural Trust, Rothamsted, U.K.) was used for all statistical analyses (analysis of variance, *P* < 0.05).

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LITERATURE CITED

Andersson, R. A., Eriksson, A. R., Heikinheimo, R., Mae, A., Pirhonen, M., Koiv, V., Hyytiäinen, H., Tuikkala, A., and Palva, E. T. 2000. Quorum sensing in the plant pathogen *Erwinia carotovora* subsp. *carotovora*: The role of *expR* (*Ecc*). Mol. Plant-Microbe Interact. 13:384-393.

Andro, T., Chambost, J. P., Kotoujansky, A., Cattaneo, J., Bertheau, Y., Barras, F., van Gijsegem, F., and Coleno, A. 1984. Mutants of *Erwinia chrysanthemi* defective in secretion of pectinase and cellulase. J. Bacteriol. 160:1199-1203.

Bailey, B. A. 1995. Purification of a protein from culture filtrates of *Fusarium oxysporum* that induces ethylene and necrosis in leaves of *Erythroxylum coca*. Phytopathology 85:1250-1255.

Bailey, B. A., Apel-Birkhold, P. C., and Luster, D. G. 2002. Expression of *NEP1* by *Fusarium oxysporum* f. sp. *erythroxyl* after gene replacement and overexpression using polyethylene glycol-mediated transformation. Phytopathology 92:833-841.

Bainton, N. J., Stead, P., Chhabra, S. R., Bycroft, B. W., Salmond, G. P. C., Stewart, G. S. A. B., and Williams, P. 1992. *N*-(3-Oxohexanoyl)-L-homoserine lactone regulates carbapenem antibiotic production in *Erwinia carotovora*. Biochem. J. 288:997-1004.

Barras, F., van Gijsegem, F., and Chatterjee, A. K. 1994. Extracellular enzymes and pathogenesis of soft-rot *Erwinia*. Annu. Rev. Phytopathol. 32:201-234.

Bell, K. S., Sebaihia, M., Pritchard, L., Holden, M. T. G., Hyman, L. J., Holeva, M. C., Thomson, N. R., Bentley, S. D., Churcher, L. J. C., Mungall, K., Atkin, R., Bason, N., Brooks, K., Chillingworth, T., Clark, K., Doggett, J., Fraser, A., Hance, Z., Hauser, H., Jagels, K., Moule, S., Norbertczak, H., Ormond, D., Price, C., Quail, M. A., Sanders, M., Walker, D., Whitehead, S., Salmond, G. P. C., Birch, P. R. J., and Toth, I. K. 2004. Genome sequence of the enterobacterial phytopathogen *Erwinia carotovora* subsp. *atroseptica* and characterization of virulence factors. Proc. Natl. Acad. Sci. U.S.A. 101:11105-11110.

Bosgelmez, G. 1999. Regulation of carbapenem antibiotic synthesis genes in *Erwinia carotovora* subspecies *carotovora*. Ph.D. thesis, University of Cambridge, UK.

Brickman, E., and Beckwith, J. 1975. Analysis of the regulation of *Escherichia coli* alkaline phosphatase synthesis using deletions and Φ80 transducing phages. J. Mol. Biol. 96:307-316.

Chatterjee, A., Cui, Y., and Chatterjee, A. K. 2002. RsmA and the quorum-sensing signal, *N*-[3-oxohexanoyl]-L-homoserine lactone, control the levels of *rsmB* RNA in *Erwinia carotovora* subsp. *carotovora* by affecting its stability. J. Bacteriol. 184:4089-95.

Chatterjee, A., Cui, Y. Y., Dumenyo, C. K., and Chatterjee, A. K. 1995. Inactivation of *rsmA* leads to overproduction of extracellular pectinases, cellulases and proteases in *Erwinia carotovora* subsp. *carotovora* in the absence of the starvation cell density signal, *N*-(3-oxohexanoyl)-L-homoserine lactone. Appl. Environ. Microbiol. 61:1959-1967.

Chen, H., Teplitski, M., Robinson, J. B., Rolfe, B. G., and Bauer, W. D. 2003. Proteomic analysis of wild-type *Sinorhizobium meliloti* responses to *N*-acyl homoserine lactone quorum-sensing signals and the transition to stationary phase. J. Bacteriol. 185:5029-5036.

Clement, J. M., Perrin, D., and Hedgpeith, J. 1982. Analysis of lambda receptor and beta-lactamase synthesis and export using cloned genes in a minicell system. Mol. Gen. Genet. 185:302-310.

Cui, Y., Chatterjee, A., Liu, Y., Dumenyo, C. K., and Chatterjee, A. K. 1995. Identification of a global repressor gene, *rsmA*, of *Erwinia carotovora* subsp. *carotovora* that controls extracellular enzymes, *N*-(3-oxohexanoyl)-L-homoserine lactone, and pathogenicity in soft-rotting *Erwinia* spp. J. Bacteriol. 177:5108-5115.

Dower, W. J., Miller, J. F., and Ragsdale, C. W. 1988. High-efficiency transformation of *Escherichia coli* by high voltage electroporation. Nucleic Acids Res. 16:6127-6145.

Eberhard, A., Burlingame, A. L., Eberhard, C., Kenyon, G. L., Nealson, K. H., and Oppenheimer, N. J. 1981. Structural identification of autoinducer of *Photobacterium fischeri* luciferase. Biochemistry 20:2444-2449.

Fellbrich, G., Romanski, A., Varet, A., Blume, B., Brunner, F., Engelhardt, S., Felix, G., Kemmerling, B., Krzymowska, M., and Nurnberger, T. 2002. NPP1, a *Phytophthora*-associated trigger of plant defense in parsley and *Arabidopsis*. Plant J. 32:375-390.

Hankin, L., and Anagnostakis, S. L. 1975. The use of solid media for the detection of enzyme production by fungi. Mycologia 67:597-607.

Harris, S. J., Shih, Y. L., Bentley, S. D., and Salmond, G. P. C. 1998. The *hexA* gene of *Erwinia carotovora* encodes a LysR homologue and regulates motility and the expression of multiple virulence determinants. Mol. Microbiol. 28:705-717.

Hinton, J. C., and Salmond, G. P. C. 1987. Use of *TnphoA* to enrich for extracellular enzyme mutants of *Erwinia carotovora* subspecies *carotovora*. Mol. Microbiol. 1:381-6.

Jones, S., Yu, B., Bainton, N. J., Birdsall, M., Bycroft, B. W., Chhabra, S. R., Cox, A. J. R., Golby, P., Reeves, P. J., Stephens, S., Winson, M. K., Salmond, G. P. C., Stewart, G., and Williams, P. 1993. The Lux autoinducer regulates the production of exoenzyme virulence determinants in *Erwinia carotovora* and *Pseudomonas aeruginosa*. EMBO (Eur. Mol. Biol. Organ.) J. 12:2477-2482.

Jores, J., Appel, B., and Lewin, A. 2003. Cloning and molecular characterization of a unique hemolysin gene of *Vibrio pommerensis* sp. nov.: Development of a DNA probe for the detection of the hemolysin gene and its use in identification of related *Vibrio* spp. from the Baltic sea.

- FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett. 229:223-229.
- Kim, Y. R., Lee, S. E., Kim, C. M., Kim, S. Y., Shin, E. K., Shin, D. H., Chung, S. S., Choy, H. E., Progulske-Fox, A., Hillman, J. D., Handfield, M., and Rhee, J. H. 2003. Characterisation and pathogenic significance of *Vibrio vulnificus* antigens preferentially expressed in septicemic patients. *Infect. Immun.* 71:5461-5471.
- Klement, Z. 1963. Rapid detection of pathogenicity of phytopathogenic pseudomonads. *Nature* 199:299-300.
- Koch, W., Wagner, C., and Seitz, H. U. 1998. Elicitor-induced cell death and phytoalexin synthesis in *Daucus carota* L. *Planta* 206:523-532.
- Koiv, V., and Mae, A. 2001. Quorum sensing controls the synthesis of virulence factors by modulating *rsmA* gene expression in *Erwinia carotovora* subsp. *carotovora*. *Mol. Genet. Genom.* 265:287-292.
- Liu, Y., Cui, Y., Mukherjee, A., and Chatterjee, A. K. 1998. Characterization of a novel RNA regulator of *Erwinia carotovora* subsp. *carotovora* that controls production of extracellular enzymes and secondary metabolites. *Mol. Microbiol.* 29:219-234.
- Manoil, C., and Beckwith, J. 1985. *TnphoA*: A transposon probe for protein export signals. *Proc. Natl. Acad. Sci. U.S.A.* 82:8129-33.
- McClean, K. H., Winson, M. K., Fish, L., Taylor, A., Chhabra, S. R., Camara, M., Daykin, M., Lamb, J. H., Swift, S., Bycroft, B. W., Stewart, G. S. A. B., and Williams, P. 1997. Quorum sensing and *Chromobacterium violaceum*: Exploitation of violacein production and inhibition for the detection of *N*-acylhomoserine lactones. *Microbiology* 143:3703-3711.
- McGowan, S., Sebaihia, M., Jones, S., Yu, B., Bainton, N., Chan, P. F., Bycroft, B., Stewart, G. S. A. B., Williams, P., and Salmond, G. P. C. 1995. Carbapenem antibiotic production in *Erwinia carotovora* is regulated by CarR, a homolog of the Lux transcriptional activator. *Microbiology* 141:541-550.
- Mukherjee, A., Cui, Y. Y., Liu, Y., Dumenyo, C. K., and Chatterjee, A. K. 1996. Global regulation in *Erwinia* species by *Erwinia carotovora rsmA*, a homologue of *Escherichia coli csrA*: Repression of secondary metabolites, pathogenicity and hypersensitive reaction. *Microbiology* 142:427-434.
- Muller, J., Walter, F., van Dijk, J. M., and Bahnke, D. 1992. Suppression of the growth and export defects of an *Escherichia coli secA* (Ts) mutant by a gene cloned from *Bacillus subtilis*. *Mol. Gen. Genet.* 235:89-96.
- Nachin, L., and Barras, F. 2000. External pH: An environmental signal that helps to rationalize *pel* gene duplication in *Erwinia chrysanthemi*. *Mol. Plant-Microbe Interact.* 13:882-886.
- Nelson, A. J., Apel-Birkhold, P. C., and Bailey, B. A. 1998. Sequence announcement. *Plant Mol. Biol.* 38:911-912.
- Neu, H. C., and Heppel, L. A. 1965. The release of enzymes from *Escherichia coli* osmotic shock and during the formation of spheroplasts. *J. Biol. Chem.* 240:3685-3692.
- Park, S. R., Kim, M. K., Kim, J. O., Bae, D. W., Cho, S. J., Cho, Y. U., and Yun, H. D. 2000. Characterization of *Erwinia chrysanthemi* PY35 *cel* and *pel* gene existing in tandem and rapid identification of their gene products. *Biochem. Biophys. Res. Commun.* 268:420-425.
- Parker, W. L., Rathnum, M. L., Wells, J. S., Trejo, W. H., Principe, P. A., and Sykes, R. B. 1982. SQ 27,860, a simple carbapenem produced by species of *Serratia* and *Erwinia*. *J. Antibiot.* 35:653-660.
- Pemberton, C. L., and Salmond, G. P. C. 2004. The Nep1-like proteins—A growing family of microbial elicitors of plant necrosis. *Mol. Plant Pathol.* 5:353-359.
- Perombelon, M. C. M. 2002. Potato diseases caused by soft rot erwinias: An overview of pathogenesis. *Plant Pathol.* 51:1-12.
- Pirhonen, M., Flego, D., Heikinheimo, R., and Palva, E. T. 1993. A small diffusible signal molecule is responsible for the global control of virulence and exoenzyme production in the plant pathogen *Erwinia carotovora*. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:2467-2476.
- Qutob, D., Kamoun, S., and Gijzen, M. 2002. Expression of a *Phytophthora sojae* necrosis-inducing protein occurs during transition from biotrophy to necrotrophy. *Plant J.* 32:361-373.
- Rivet, M. M. 1998. Investigation into the regulation of exoenzyme production in *Erwinia carotovora* subspecies *carotovora*. Ph.D. thesis, University of Warwick, U.K.
- Roy, C., Kester, H., Visser, J., Shevchik, V., Hugouvieux-Cotte-Pattat, N., Robert-Baudouy, J., and Benen, J. 1999. Modes of action of five different endopeptidase lyases from *Erwinia chrysanthemi* 3937. *J. Bacteriol.* 181:3705-3709.
- Salanoubat, M., Genin, S., Artiguenave, F., Gouzy, J., Mangelot, S., Arlat, M., Billault, A., Brottier, P., Camus, J. C., Cattolico, L., Chandler, M., Choise, N., Claudel-Renard, C., Cunnac, S., Demange, N., Gaspin, C., Lavie, M., Moisan, A., Robert, C., Saurin, W., Schiex, T., Siguier, P., Thebault, P., Whalen, M., Wincker, P., Levy, M., Weissenbach, J., and Boucher, C. A. 2002. Genome sequence of the plant pathogen *Ralstonia solanacearum*. *Nature* 415:497-502.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, U.S.A.
- Schuster, M., Lostroh, C. P., Ogi, T., and Greenberg, E. P. 2003. Identification, timing, and signal specificity of *Pseudomonas aeruginosa* quorum-controlled genes: A transcriptome analysis. *J. Bacteriol.* 185:2066-2079.
- Shevchik, V. E., Kester, H. C., Benen, J. A., Visser, J., Robert-Baudouy, J., and Hugouvieux-Cotte-Pattat, N. 1999. Characterization of the exopolysaccharide lyase PelX of *Erwinia chrysanthemi* 3937. *J. Bacteriol.* 181:1652-1663.
- Swift, S., Winson, M. K., Chan, P. F., Bainton, N. J., Birdsall, M., Reeves, P. J., Rees, C. E. D., Chhabra, S. R., Hill, P. J., Throup, J. P., Bycroft, B. W., Salmond, G. P. C., Williams, P., and Stewart, G. 1993. A novel strategy for the isolation of LuxI homologues: Evidence for the widespread distribution of a LuxR:LuxI superfamily in enteric bacteria. *Mol. Microbiol.* 10:511-520.
- Thanassi, D. G., Saulino, E. T., Lombardo, M. J., Roth, R., Heuser, J., and Hultgren, S. J. 1998. The PapC usher forms an oligomeric channel: Implications for pilus biogenesis across the outer membrane. *Proc. Natl. Acad. Sci. U.S.A.* 95:3146-3151.
- Toth, I. K., Perombelon, M., and Salmond, G. P. C. 1993. Bacteriophage ϕ KP mediated generalized transduction in *Erwinia carotovora* subsp. *carotovora*. *J. Gen. Microbiol.* 139:2705-2709.
- Toth I. K., Mulholland, V., Cooper, V., Bentley, S., Shih, Y. L., Perombelon, M. C. M., and Salmond, G. P. C. 1997. Generalised transduction in the blackleg pathogen *Erwinia carotovora* subsp. *atroseptica* by bacteriophage ϕ M1. *Microbiology* 143:2433-2438.
- Thomson, N. R., Thomas, J. D., and Salmond, G. P. C. 1999. Virulence determinants in the bacterial phytopathogen *Erwinia*. *Methods Microbiol.* 29:347-426.
- Wagner, V. E., Bushnell, D., Passador, L., Brooks, A. I., and Iglewski, B. H. 2003. Microarray analysis of *Pseudomonas aeruginosa* quorum-sensing regulons: Effects of growth phase and environment. *J. Bacteriol.* 185:2080-2095.
- Walker, D., Reeves, P. J., and Salmond, G. P. C. 1994. The major secreted cellulase, CelV, of *Erwinia carotovora* subsp. *carotovora* is an important virulence factor. *Mol. Plant-Microbe Interact.* 7:425-431.
- Welch, M., Todd, D. E., Whitehead, N. A., McGowan, S. J., Bycroft, B. W., and Salmond, G. P. C. 2000. *N*-Acyl homoserine lactone binding to the CarR receptor determines quorum-sensing specificity in *Erwinia*. *EMBO (Eur. Mol. Biol. Organ.) J.* 19:631-641.
- Whitehead, N. A., Barnard, A. M. L., Slater, H., Simpson, N. J. L. S., and Salmond, G. P. C. 2001. Quorum-sensing in Gram-negative bacteria. *FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Rev.* 25:365-404.
- Wilmes-Riesenberg, M. R., and Wanner, B. L. 1992. *TnphoA* and *TnphoA'* elements for making and switching fusions for study of transcription, translation, and cell surface localization. *J. Bacteriol.* 174:4558-4575.
- Xi, C., Lambrecht, M., Vanderleyden, J., and Michiels, J. 1999. Bi-functional *gfp+* and *gusA*-containing mini-Tn5 transposon derivatives for combined gene expression and bacterial localization studies. *J. Microbiol. Methods* 35:85-92.

AUTHOR-RECOMMENDED INTERNET RESOURCES

European Bioinformatics Institute website: www.ebi.ac.uk
 PSORT server: psort.nibb.ac.jp
 Scottish Crop Research Institute *Erwinia* genome sequence webpage: www.scri.sari.ac.uk/erwinia
 The Wellcome Trust Sanger Institute website: www.sanger.ac.uk