Involvement of *N*-acylhomoserine Lactones Throughout Plant Infection by *Erwinia carotovora* subsp. *atroseptica* (*Pectobacterium atrosepticum*)

Bruno Smadja,¹ Xavier Latour,¹ Denis Faure,² Sylvie Chevalier,¹ Yves Dessaux,² and Nicole Orange¹

¹Laboratoire de Microbiologie du Froid—UPRES 2123, Université de Rouen, 55 rue Saint-Germain, 27000 Evreux, France;

²Institut des Sciences du Végétal—UPR 2355, Centre National de la Recherche Scientifique, Avenue de la Terrasse, 91198 Gif-sur-Yvette, France

Submitted 10 February 2004. Accepted 13 July 2004.

Erwinia carotovora subsp. *atroseptica* is responsible for potato blackleg disease in the field and tuber soft rot during crop storage. The process leading to the disease occurs in two phases: a primary invasion step followed by a maceration step. Bacteria-to-bacteria communication is associated with a quorum-sensing (QS) process based on the production of *N*-acylhomoserine lactones (HSL). The role of HSL throughout plant infection was analyzed. To this purpose, HSL produced by a specific *E. carotovora* subsp. *atroseptica* wild-type strain, which was particularly virulent on potato, were identified. A derivative of this strain that expressed an HSL lactonase gene and produced low amounts of HSL was generated. The comparison of these strains allowed the evaluation of the role of HSL and QS in disease establishment and development. Bacterial growth and motility; activity of proteins secreted by type I, II, and III systems; and hypersensitive and maceration reactions were evaluated. Results indicated that HSL production and QS regulate only those traits involved in the second stage of the host plant infection (i.e., tissue maceration) and hypersensitive response in nonhost tobacco plants. Therefore, the use of QS quenching strategies for biological control in *E. carotovora* subsp. *atroseptica* cannot prevent initial infection and multiplication of this pathogen.

Additional keywords: extracellular enzymes, *hrp* genes, *Nicotiana tabacum*, *Solanum tuberosum*.

The pectinolytic *Erwinia* spp. are soft-rotting gram-negative bacteria that cause severe economic damage on various crops. The process underlying the development of the disease can be broken down into several steps, for which the chronology of events is particularly significant (Byers et al. 2002; Mäe et al. 2001). Initially, bacteria are present in a latent state in infection areas such as plant wounds or lenticels (Scott et al. 1996). They begin to multiply actively to reach a critical cell density (i.e., between 10^7 and 10^8 cells/g of tissue) (Pérombelon 2002; Pérombelon and Salmond 1995). Some *Erwinia* strains pro-

duce the antibiotic carbapenem, a feature that could facilitate the colonization of their ecological niches (Axelrood et al. 1988; Whitehead et al. 2002). In the second phase, the bacteria degrade plant tissues by producing massive amounts of numerous lytic extracellular enzymes (Barras et al. 1994). The production of carbapenem and of lytic enzymes is controlled in part by a quorum-sensing (QS) process that relies upon the production of *N*-acylhomoserine lactones (HSL) (Bainton et al. 1992; Jones et al. 1993; Pirhonen et al. 1993). As a consequence, the production of these enzymes and antibiotics is triggered in a synchronous way and only when the population density of the pathogen has reached a threshold value; hence the word “quorum” (Whitehead et al. 2001).

Among pectinolytic bacteria, those of the subspecies *Erwinia carotovora* subsp. *atroseptica* are responsible for blackleg, a typical stem infection of potato (*Solanum tuberosum*) in the field. They also are responsible for tuber soft rot during crop storage. *E. carotovora* subsp. *atroseptica* causes important losses within cool temperate regions such as northwestern Europe, where potato traditionally has been grown (Pérombelon and Salmond 1995; Priou and Jouan 1996). Based on host specificity and physiological, biochemical, serological, and genotypic characteristics, *E. carotovora* subsp. *atroseptica* can be delineated from the other macerating *Erwinia* spp., and can be described as a species novae *Pectobacterium atrosepticum* (Gardan et al. 2003; Hauben et al. 1998; Smadja et al. 2004). Although the specific role played by HSL production and QS regulation on pathogenicity and bacterial metabolism has been studied in other pectinolytic species, limited data are available for this redoubtable pathogen of potato (Whitehead et al. 2001).

To address this question, a novel tool was used. Indeed, the recent discovery of enzymes that degrade specifically HSL (Carlier et al. 2003; Dong et al. 2000; Leadbetter and Greenberg, 2000; Lee et al. 2002; Reimann et al. 2002) offers the opportunity to identify and to perturb (to quench) the HSL-controlled traits in *E. carotovora* subsp. *atroseptica*, whether or not these are involved in the virulence of the bacteria. Using this strategy, a strain producing low amounts of HSL was constructed by the introduction of an HSL-lactonase gene into the bacterium. Various phenotypes of this strain were compared with those of its wild-type parent, which is particularly virulent on potato (Smadja et al. 2004). The analysis elucidated the specific contribution of QS regulation at the two stages of the disease. The primary invasion stage was examined by studying

Corresponding author: X. Latour; Telephone: +33 2 32291549; Fax +33 2 32291550; E-mail: xavier.latour@univ-rouen.fr

Nucleotide sequence data reported are available in the GenBank database under accession numbers AJ580601 and AJ577998 for *hrcC* and *hrpN* respectively, of *Pectobacterium atrosepticum* CFBP 6276.

i) the cellular multiplication, bacterial motility, and antibiotic production in a synthetic medium and ii) potato tuber colonization. The second stage was studied by evaluating the maceration of potato tuber tissues and the activity of enzymes involved in the maceration process, namely cellulases (Cel, EC 3.2.1.4), proteases (Prt, EC 3.4.24.-), and pectinolytic enzymes, which include pectin methylesterase (Pme, EC 3.1.1.11), pectin lyase (Pnl, EC 4.2.2.10), pectate lyases (Pel, EC 4.2.2.2), and pectate hydrolases (Peh, EC 3.2.1.15). Finally, the potential of *E. carotovora* subsp. *atroseptica* to induce a hypersensitive response (HR) was evaluated in a tobacco nonhost plant. The kinetics of *hrp* genes transcription, presumably involved in this HR and determining a type III secretion system, also were compared in the wild-type parental and modified strains, using reverse-transcription polymerase chain reaction (RT-PCR).

RESULTS

Characterization of the HSL produced by wild-type *E. carotovora* subsp. *atroseptica* and derivative strains.

Under the experimental conditions described below, *E. carotovora* subsp. *atroseptica* strain 6276 presumably produces two unsubstituted HSLs (i.e., *N*-octanoyl-L-homoserine lactone [C8-HSL] and *N*-decanoyl-L-homoserine lactone [C10-HSL]), and the substituted HSL *N*-3-oxo-octanoyl-L-homoserine lactone (3-oxo-C8-HSL) (Fig. 1). To quench HSL production by

E. carotovora subsp. *atroseptica* 6276, plasmid pME6863, which encodes the lactonase gene *aiiA* from *Bacillus* sp., and the corresponding “empty vector” pME6000 were introduced into this strain by electroporation (Table 1). The analysis of the spent culture supernatants revealed that the amounts of all HSL produced by the parental strain or the strain carrying pME6000 were substantially reduced in *E. carotovora* subsp. *atroseptica* carrying pME6863 (Fig. 1). Serial dilution of the HSL extracts showed a 1,000-fold reduction of HSL concentration in the supernatant of the strain harboring the *aiiA* lactonase gene (data not shown).

Bacterial functions affected by the quenching of HSL production in *E. carotovora* subsp. *atroseptica* 6276.

The growth rates of *E. carotovora* subsp. *atroseptica* 6276, *E. carotovora* subsp. *atroseptica* 6276(pME6000), and *E. carotovora* subsp. *atroseptica* 6276(pME6863) in polygalacturonic acid (PGA) medium were 0.329 ± 0.007 , 0.301 ± 0.014 , and 0.319 ± 0.019 h⁻¹, respectively, and were not statistically different ($P = 0.05$). Under these growth conditions, the supernatants and cells were saved and assayed for Pme, Pnl, Pel, Peh, Cel, and Prt activities. All the pectinolytic activities were lower in *E. carotovora* subsp. *atroseptica* 6276 carrying pME6863 compared with the parental strain or the strain carrying the empty cloning vector (Table 2). Similarly, the cellulolytic and proteolytic activities were lower in *E. carotovora*

Table 1. Strains and plasmids used in this study

Strain and plasmid	Relevant characteristic ^a	Reference
Bacteria		
<i>Agrobacterium tumefaciens</i> NTLR4	NT1 derivative of strain C58, carrying pZLR4 plasmid, HSL biosensor, Gm ^r	Cha et al. 1998
<i>Chromobacterium violaceum</i> CV026	Double mini Tn5 mutant derived from strain ATCC 31532, HSL biosensor, Km ^r	McClean et al. 1997
<i>Erwinia carotovora</i> subsp. <i>atroseptica</i> (Pectobacterium atrosepticum) CFBP 6276	Potato soft rot pathogen, HSL producer	Smadja et al. 2004
6276(pME6000)	<i>E. carotovora</i> subsp. <i>atroseptica</i> strain CFBP 6276 harboring pME6000 plasmid	This study
6276(pME6863)	<i>E. carotovora</i> subsp. <i>atroseptica</i> strain CFBP 6276 harboring pME6863 plasmid	This study
<i>Escherichia coli</i> DH5α	φ80lacZ ΔM15 Δ(lacZYA-argF) U169 hsdR17 recA1 endA1 thi-1, supersensitive to the β-lactams	Sambrook et al. 1989
ESS		Bainton et al. 1992
XL1-Blue	recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac (F' proAB lacIqZM15: Tn10 [Tc ^r])	Bullock et al. 1987
Plasmids		
pME6000	Broad-host-range cloning vector, Tc ^r	Maurhofer et al. 1998
pME6863	pME6000 carrying the <i>aiiA</i> gene from <i>Bacillus</i> sp. A24 under the constitutive P _{lac} promoter	Reimann et al. 2002
pMOSBlue	Broad-host-range cloning vector, Tc ^r	Bullock et al. 1987
pZLR4	traG::lacZ/traR reporter system	Cha et al. 1998

^a Gm^r, Km^r, and Tc^r indicate resistance to gentamicin, kanamycin, and tetracycline, respectively. HSL, *N*-acylhomoserine lactones; CFBP, Collection Française de Bactéries Phytopathogènes, Institut National de la Recherche Agronomique, Angers, France.

Table 2. Effect of *N*-acylhomoserine lactones on motility and enzymatic activities in *Erwinia carotovora* subsp. *atroseptica* 6276

Activity ^b	<i>E. carotovora</i> subsp. <i>atroseptica</i> ^a		
	6276	6276(pME6000) (empty vector)	6276(pME6863) (AiiA ⁺)
Motility			
Microscopic observation	+++	+++	+++
Swarming test	+++	+++	+++
Swimming test	+++	+++	+++
Enzymes activity			
Pme	+++	+++	++
Pnl	+++	+++	++
Pel	+++	+++	++
Peh	+++	+++	+
Cel	+++	+++	–
Prt	+++	+++	++

^a Activity level: +++, reference motility or enzymes activity (diameter of lysis); ++, two-thirds of reference enzymes activity; +, one-third of reference enzymes activity; –, no activity.

^b Pme, pectin methylesterase; Pnl, pectin lyase; Pel, pectate lyases; Peh, pectate hydrolases; Cel, cellulases; Prt, proteases.

subsp. *atroseptica* 6276(pME6863) than in the parental or control strains (Table 2). Pel and Prt are related to different pathways of carbon and nitrogen metabolism and are secreted by type II and I systems, respectively. Due to the documented importance of Pel and Prt activities in disease development (Liao 1989; Marits et al. 1999), the intensity of each of these activities was subsequently determined during bacterial growth by quantitative spectrophotometric assays. Production of Pel and Prt was statistically similar ($P = 0.05$) in *E. carotovora* subsp. *atroseptica* 6276 and its derivative carrying pME6000. In contrast, production of Pel and Prt were reduced $\approx$ fivefold and twofold, respectively, in the strain carrying pME6863 (Fig. 2).

No difference in the motility of strains *E. carotovora* subsp. *atroseptica* 6276, *E. carotovora* subsp. *atroseptica* 6276(pME6000), and *E. carotovora* subsp. *atroseptica* 6276(pME6863), as examined under the microscope and on agar media, was detected (Table 2). *E. carotovora* subsp. *atroseptica* 6276 and its derivatives did not produce detectable carbapenem under the assay conditions: no inhibition zone was observed on a lawn of *Escherichia coli* strain ESS that is supersensitive to the β -lactams, in contrast with the positive inhibition assays performed with this indicator bacteria and ampicillin disks.

Consequence of the quenching of HSL production on tuber soft rot.

The potato tuber test allowed the determination of both the bacterial population density in tissues and the weight of macerated tissue as a function of time after tuber inoculation. In planta, growth of *Erwinia carotovora* subsp. *atroseptica* 6276 and *E. carotovora* subsp. *atroseptica* 6276(pME6000) were

Fig. 1. Identification of *N*-acylhomoserine lactones (HSL) in spent supernatants of *Erwinia carotovora* subsp. *atroseptica* 6276 and its derivatives. Ethyl acetate extracts of spent culture supernatant were spotted onto C_{18} reversed-phase thin-layer plates that were developed with methanol:water (60:40, vol/vol). The plates were overlaid with the sensor strain *Agrobacterium tumefaciens* NTLR4. Standards were spotted in lane 1 as a mixture of unsubstituted HSL (i.e., C6-HSL, C7-HSL, C8-HSL, and C10-HSL) and in lane 5 as a mixture of substituted HSL (i.e., 3-oxo-C6-HSL and 3-oxo-C8-HSL). Other lanes were extracts from: lane 2, *E. carotovora* subsp. *atroseptica* 6276; lane 3, *E. carotovora* subsp. *atroseptica* 6276(pME6000) (empty vector), and lane 4, *E. carotovora* subsp. *atroseptica* 6276(pME6863) (AiiA⁺).

identical statistically ($P = 0.05$) to that of the strain carrying the plasmid encoding AiiA (Fig. 3A). In planta, growth appeared to be exponential, with a maximum population density of 10^9 CFU g^{-1} of macerated tissue, observed after 3 days of incubation. The strain expressing the *aaiA* gene, however, showed a reduced ability to macerate tissues compared with the parental and control strains (Fig. 3B and C). The amounts of macerated tissues were $\approx$ twofold lower when the *E. carotovora* subsp. *atroseptica* strain contained the lactonase gene: in this case, a slight and uncolored maceration was observed.

Fig. 2. Quenching *N*-acylhomoserine lactones production and consequence on growth and extracellular enzyme activities of *Erwinia carotovora* subsp. *atroseptica* (*Eca*) strain 6276 and its derivatives. The strains used here were *Eca* 6276, *Eca* 6276(pME6000) carrying the empty plasmid vector, and *Eca* 6276(pME6863) that expresses the lactonase AiiA. **A**, Growth of *Eca* 6276 (■), *Eca* 6276(pME6000) (▲), and *Eca* 6276(pME6863) (●), grown in the minimum medium supplemented with polygalacturonic acid at 24°C; **B**, pectate lyase (Pel) activity; and **C**, protease (Prt) activity in *Eca* 6276 (■), *Eca* 6276(pME6000) (▲), and *Eca* 6276(pME6863) (●) in cultures supernatants collected during the growth. Each value represents the mean of three experiments. Bars indicate the standard deviation.

Fig. 3. Quenching *N*-acylhomoserine lactones production and consequence on invasion and soft rot tuber due to *Erwinia carotovora* subsp. *atroseptica* (*Eca*) strain 6276 and its derivatives. Disease was evaluated on tuber potato after local inoculation of *Eca* strains. **A**, Cell density of bacteria expressed as CFU g⁻¹ of macerated tissues is shown for *Eca* 6276 (■), *Eca* 6276(pME6000) (▲), and *Eca* 6276(pME6863) (●). **B**, Weight of macerated tissue is shown for *Eca* 6276 (□), *Eca* 6276(pME6000) (▲), and *Eca* 6276(pME6863) (○). **C**, Visual observation of potato tuber maceration induced by *Eca* 6276(pME6000) (line 1), *Eca* 6276(pME6863) (line 2), and the sterile control consisting in 0.9% NaCl (line 3). Pictures were taken 14 days after inoculation.

Consequence of the quenching of HSL production on the nonhost HR and *hrp* gene transcription.

E. carotovora subsp. *atroseptica* 6276 induced an HR-like response upon inoculation into nonhost plants such as tobacco. The strain *E. carotovora* subsp. *atroseptica* 6276 and that carrying the empty vector triggered symptoms which resemble those of a typical HR and differed from the maceration symptoms (Fig. 4A and B). This response appeared 16 and 24 h following inoculation on *Nicotiana tabacum* cv. Xanthi and *N. benthamiana*, respectively, when strains were cultivated in PGA medium and inoculated at high density. No reaction was induced by strain *E. carotovora* subsp. *atroseptica* 6276(pME6863) under the same conditions (Fig. 4C).

The internal segments of the putative *hrcC* (516 bp) and *hrpN* (274 bp) genes of *E. carotovora* subsp. *atroseptica* 6276 were cloned and sequenced. BLASTP sequence comparisons

of the 516-bp *hrcC* fragment translated in silico, that corresponds to a peptide of 172 amino acid (aa) residues, showed 97, 60, and 60% identity with the HrcC protein of *E. carotovora* subsp. *carotovora* SCC1 (Rantakari et al. 2001), *E. chrysanthemi* EC16 (Bauer et al. 1995), and *E. amylovora* Ea321 (Kim et al. 1997), respectively. The 274-bp *hrpN* fragment, encoding a putative 91-aa sequence, showed 95, 84, and 76% identity with the harpin of *E. carotovora* subsp. *carotovora* SCC1 (Rantakari et al. 2001), *E. chrysanthemi* EC16 (Bauer et al. 1995), and *E. amylovora* (Wei et al. 1992), respectively. The kinetics of the transcription of both *hrcC*_{Eca} and *hrpN*_{Eca} genes were determined by semiquantitative RT-PCR experiments. Total RNAs were extracted from *E. carotovora* subsp. *atroseptica* 6276, *E. carotovora* subsp. *atroseptica* 6276(pME6000), and *E. carotovora* subsp. *atroseptica* 6276(pME6863) cultured either in PGA medium or in potatoes

Fig. 4. Elicitation of a hypersensitive response in tobacco leaves (*Nicotiana tabacum* cv. Xanthi) by *Erwinia carotovora* subsp. *atroseptica* 6276 and its derivative strains. Leaf panels were infiltrated with cells of strain **A**, *Eca* 6276, **B**, *Eca* 6276(pME6000), and **C**, *Eca* 6276(pME6863) at a concentration of 2×10^9 CFU ml⁻¹ in 0.9% NaCl. Pictures were taken 16 h after infiltration.

Fig. 5. Quenching *N*-acylhomoserine lactones production and consequence on *hrp* gene transcription in *Erwinia carotovora* subsp. *atroseptica* (*Eca*) 6276 and its derivative strains. Transcription of **A** and **B**, *hrcC*_{Eca} and **C** and **D**, *hrpN*_{Eca} were determined by reverse-transcription polymerase chain reaction experiments with cells harvested from **A** and **C**, PGA medium or **B** and **D**, potato tubers at three different stage of growth: exponential phase (8 or 12 h), late-exponential phase (16 or 48 h), and stationary growth phase (24 or 96 h). Results were expressed as a ratio: *hrcC*_{Eca} or *hrpN*_{Eca} transcripts versus 16S transcripts.

tubers and at three different phases of the growth kinetics (i.e., mid-exponential, late-exponential, and stationary phase). The transcription of *hrc*_{Eca} appeared similar within the three strains: the amounts of transcripts increased from the end of the exponential phase to the stationary phase of growth only in the in vitro assays (Fig. 5A and B). The transcription of *hrpN*_{Eca} was quite similar in the strains *E. carotovora* subsp. *atroseptica* 6276 and in that carrying the empty vector: it was stimulated from the end of the exponential growth. In contrast and, remarkably, strain *E. carotovora* subsp. *atroseptica* 6276(pME6863) maintained the same basal transcription level throughout growth in vivo and in planta (Fig. 5C and D).

DISCUSSION

E. carotovora subsp. *atroseptica* strain 6276 is a virulent pathogen of potato typical of the psychrotolerant new species *Pectobacterium atrosepticum* (Gardan et al. 2003; Smadja et al. 2004). This bacterium produces at least three HSLs, most likely 3-oxo-C8-HSL, C8-HSL, and C10-HSL. In the related macerating *Erwinia* spp., 3-oxo-C6-HSL is the only—or the main—HSL for which a biological role has been demonstrated (Chatterjee et al. 1995; Jones et al. 1993; Nasser et al. 1998; Pirhonen et al. 1993). To study the involvement of HSL and, hence, that of QS in pathogenicity-related traits, an *E. carotovora* subsp. *atroseptica* strain 6276 which expresses the lactonase AiiA from *Bacillus* sp. strain A24 (Reimann et al. 2002) was constructed. In the culture supernatants of such a strain, the concentrations of all HSL were efficiently reduced as the result of the lactonase activity, allowing several HSL-regulated functions to be identified during infection of host (potato) and nonhost (tobacco) plants.

The two major steps of infection were clearly distinguished when *E. carotovora* subsp. *atroseptica* 6276 was inoculated to potato tubers. First, the wild-type bacteria grow to a population density of 10^9 CFU g⁻¹ in 48 h. Second, once at a high population density, bacteria trigger maceration of the plant tissues around the infection area. The colonization of the infection area requires efficient bacterial multiplication, because only a high population density will allow the bacteria to overcome the defense mechanisms elaborated by the plant (Toth et al. 2003). Bacterial growth can be modulated by QS via HSL production in soilborne or plant-related bacteria (Batchelor et al. 1997; Thorne and Williams 1999). According to this study, it appears that a marked reduction of the concentration of HSL has no effect on either the growth rate or the growth phase characteristics. Associated with multiplication, bacterial motility contributes to the infection process in various human (Josenhans and Suerbaum 2002) and plant (Harris et al. 1998; Moens and Vanderleyden 1996; Toth et al. 1999) pathogens, including *E. carotovora* subsp. *atroseptica* (Mulholland et al. 1993). Although the QS controls motility amongst different bacterial genus (Atkinson et al. 1999; Eberl et al. 1999; Reimann et al. 2002), no obvious consequence of the degradation of the QS signals was observed on the motility of the *E. carotovora* subsp. *atroseptica* strain expressing the *aiiA* gene.

The direct consequence of the dramatic fall of the concentration of the QS signal molecules is a reduction of the maceration capability of the *E. carotovora* subsp. *atroseptica* strain expressing the *aiiA* gene, assessed on potato tubers. Because there was no statistical difference ($P = 0.05$) in the growth of *E. carotovora* subsp. *atroseptica* 6276 and that of its derivatives expressing or not expressing the *aiiA* gene, it appears that the reduced maceration is not related to an alteration of the bacterial ability to grow in planta but, rather, to the decreased production of the assayed extracellular enzyme activities. In agreement with this, strain *E. carotovora* subsp. *atroseptica*

6276(pME6863) was unable to cause a consequent maceration of the plant tissues even upon prolonged inoculation (12 days) of tubers (Fig. 3).

The results revealed the key role played by QS regulation in the virulence of *E. carotovora* subsp. *atroseptica*. It also illustrates the value of HSL-degrading enzymes as tools for genetic and physiological studies on QS regulation. In addition, these results confirm the interest of a QS-quenching procedure for biological control, as originally proposed by Dong and associates (2000) and recently evaluated by Molina and associates (2003) and Uroz and associates (2003). In this respect, HSL hydrolysis (or more generally the “destruction” of the QS signals) will not prevent the establishment of a high inoculum at the infection sites. This observation should be taken into account when considering the development of the relevant biological control procedures, because contaminated tubers free of disease symptoms might be sown and possibly certified. For instance, a possible reactivation of maceration process could rapidly spoil the crop upon storage.

In addition to their interaction with compatible hosts, *Erwinia* strains induce HR in several plants. Infiltration of tobacco leaves showed that *E. carotovora* subsp. *atroseptica* 6276 triggered HR on tobacco. Using a targeted sample sequencing approach, Bell and associates (2002) have identified 25 *hrp* genes in *E. carotovora* subsp. *atroseptica*. However, their role in pathogenicity or induction of HR was not evaluated. *E. carotovora* subsp. *atroseptica* strain 6276 possesses *hrc*_{Eca} and *hrpN*_{Eca} genes, which present a clear sequence homology with the *hrcC* and *hrpN* genes of other *Erwinia* spp. The product of the *hrcC* gene is involved in *E. carotovora* subsp. *carotovora* virulence and belongs to a series of proteins, forming a type III secretion apparatus, which permits the transfer of proteins to the host cell (Collmer and Beer 1998; Deng et al. 1998; Rantakari et al. 2001). The HrpN protein is an elicitor responsible for the induction of HR by *E. carotovora* subsp. *carotovora* and *E. chrysanthemi* strains in nonhost plants (Bauer et al. 1995; Cui et al. 1996; Mukherjee et al. 1997). RT-PCR assays allowed the determination of the kinetics of the transcription of *hrcC*_{Eca} and *hrpN*_{Eca}, both in vitro and in planta. The only differential transcription activity observed was that of *hrpN*_{Eca}, both in vitro and in planta. In the *E. carotovora* subsp. *atroseptica* strain producing limited amounts of QS molecules, the transcription activity of *hrcC*_{Eca} remained constant whatever the time point or population density. This pattern of expression of *hrpN*_{Eca} is very similar to that of the production of the extracellular enzymes, which also appears to be undercontrolled by QS via HSL production in vitro and in planta. This HSL-dependent expression of *hrpN*_{Eca} is consistent with the absence of an HR in tobacco plants inoculated with *E. carotovora* subsp. *atroseptica* 6276(pME6863). This observation suggests that the putative harpin, HrpN_{Eca}, could be regulated in an HSL-dependent manner. In contrast, this process does not seem to control HrcC_{Eca} production, a putative component of the type III secretion system, presumably pre-existing before the harpin release. Nevertheless, in spite of the presumable role of HrpN_{Eca} in the HR, it is possible that the reduced levels of lytic enzymes, for example, or the expression of other *hrp* genes are responsible for the loss of the HR in tobacco.

This work provides evidence in support of the view that QS via the production of HSL during plant infection does not regulate the growth and motility of *E. carotovora* subsp. *atroseptica* during initial infection events. In contrast, QS regulation is involved in the control of the maceration process leading to the development of disease. Therefore, QS regulation seems to be involved in the steps following plant colonization. As a consequence, HSL in *E. carotovora* subsp. *atroseptica* may not be a

regulator of genes involved in plant invasion but, rather, would regulate the production of lytic enzymes and elicitors at a time when plant defenses can be overwhelmed. The HSL control of *hrpN* and *pel* genes expression seem not to be ubiquitous in pectinolytic erwiniae. It has been observed in *E. carotovora* subsp. *carotovora* (Mukherjee et al. 1997) and *E. carotovora* subsp. *atroseptica* (this study), but not in strains belonging to *E. chrysanthemi*, to whom an alternative QS molecule signal (AI-2) would be implied (Ham et al. 2004; Nasser et al. 1998). The mechanism that links HSL synthesis and *hrpN*_{Eca} expression remains to be elucidated.

MATERIALS AND METHODS

Bacterial strains and plasmids.

The characteristics of bacterial strains and plasmids are presented in Table 1. *Escherichia coli* strain DH5 α (GIBCO BRL, Cheshire, UK) was used for plasmid maintenance. The broad-host-range cloning vector pME6000 and the plasmid pME6863 have been transferred into *Erwinia carotovora* subsp. *atroseptica* strain CFBP 6276 (Smadja et al. 2004) by electroporation (Sambrook et al. 1989). The biosensor strains *Chromobacterium violaceum* CV026 and *Agrobacterium tumefaciens* NTLR4 were used for the detection of QS signal molecules.

Media, culture conditions.

E. carotovora subsp. *atroseptica* 6276 and its derivatives containing the appropriate plasmid (pME6000 or pME6863) were maintained on Luria-Bertani agar (LBA) plates containing tetracycline (10 $\mu\text{g ml}^{-1}$) when appropriate. Bacteria also were grown in PGA minimal medium supplemented with polygalacturonic acid 0.4% (wt/vol) (Sigma-Aldrich, St. Louis) as described elsewhere (Smadja et al. 2004). *E. carotovora* subsp. *atroseptica* strains were grown in liquid medium in Erlenmeyer flasks under rotary shaking (180 rpm) at 24°C. For enzyme assays with those strains, antibiotics were omitted. The preparation of extracellular enzymatic supernatants was made as described by Smadja and associates (2004). The filtered supernatants were stored at -20°C. *Escherichia coli* strains were grown in Luria-Bertani (LB) medium supplemented with ampicillin (100 $\mu\text{g ml}^{-1}$) and tetracycline (10 $\mu\text{g ml}^{-1}$), and maintained on LBA supplemented with ampicillin, tetracycline, iso-propyl-thio-galactoside (IPTG, 24 $\mu\text{g ml}^{-1}$), and 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-Gal, 40 $\mu\text{g ml}^{-1}$) when necessary. *C. violaceum* strain CV026 was grown on LB broth and maintained on LBA supplemented with kanamycin (50 $\mu\text{g ml}^{-1}$). *A. tumefaciens* strain NTLR4 was grown on AB minimal medium plus 0.2% (wt/vol) mannitol (ABM) or ABM agar (ABM supplemented with agar, 15 g liter⁻¹) (Chilton et al. 1974) supplemented with gentamicin (25 $\mu\text{g ml}^{-1}$). Both biosensor strains were cultivated at 28°C, whereas *E. coli* strains were grown at 37°C, under orbital shaking (180 rpm) if necessary.

HSL standards.

All HSL were purchased from Sigma-Aldrich (St. Louis) except 3-oxo-C8-HSL, which was kindly provided by Paul Williams (University of Nottingham, U.K.). The synthetic standards were stored at -20°C and standard stock solutions were prepared in high-performance liquid chromatography (HPLC)-grade ethyl acetate.

Streak plate assays for detection of HSL.

Screening for HSL production was performed on solid medium using a streak plate assay, as described by Piper and associates (1993). The biosensors *C. violaceum* CV026 and *A. tumefaciens* NTLR4, grown on LB broth or on ABM supple-

mented with X-Gal (40 $\mu\text{g ml}^{-1}$), respectively, were used to detect HSL.

Extraction of supernatants and HSL assays.

The sterilized supernatants (1 ml) were extracted twice with equal volumes of ethyl acetate. The extracts were treated with anhydrous magnesium sulfate and evaporated to dryness (modified from Shaw et al. 1997). Residues from 1-ml supernatants were dissolved in 50 to 100 μl of HPLC-grade ethyl acetate and stored at -20°C.

Components in the ethyl acetate extracts were spotted onto C₁₈ reversed-phase thin-layer chromatography (TLC) Silicagel (Whatman, Maidstone, U.K.) plates and separated with methanol-water (60:40, vol/vol), as described by Shaw and associates (1997). HSL were detected by overlaying the TLC with the indicator strains essentially as indicated by Cha and associates (1998).

Motility assays.

Motility of wild-type *E. carotovora* subsp. *atroseptica* and its derivatives was observed with a phase-contrast microscope. The swarming ability of these strains was assayed on tryptone swarm agar (TSA) plates according to Harris and associates (1998). The swimming ability of these strains was assayed on 0.3% agar plates according to a modification of Köhler and associates (2000). All plates were incubated at 24°C.

Antibiotic production.

Cultures of *E. carotovora* subsp. *atroseptica* strains entering the early stationary growth phase (10 μl) were spotted onto a top agar lawn of *Escherichia coli* strain ESS, supersensitive to the β -lactams (Bainton et al. 1992) on LBA medium. A positive reaction was obtained using an ampicillin disk (10 μg).

Assays for production of extracellular enzymes.

The composition of the agarose plates for the semiquantitative assay of extracellular Pel, Peh, and Cel was as previously described (Chatterjee et al. 1995), sodium azide being omitted. Tryptone soja agar (40 g liter⁻¹) supplemented with 25% (vol/vol) skimmed milk was used for Prt detection. The composition of medium for the Pnl agarose plate assay was as follows: 1% (wt/vol) methylesterified citrus pectin (Sigma-Aldrich), 0.4 mM of EDTA, 22 mM Tris-HCl (pH 8.0), and 0.8% agarose, modified from McEvoy and associates (1990). The Pme activity was detected in a media adapted from Downie and associates (1998), which contained 0.1% (wt/vol) methylesterified citrus pectin, 1% (wt/vol) yeast extract, sodium acetate buffer (0.1 M, pH 7.0), and 0.8% agarose. For the assays, 10 to 20 μl of supernatant were applied to wells dug into the plates that were further incubated for 24 to 48 h at 28°C, except those for Prt assays, which were incubated at 37°C. For Pme detection, the agarose plate assays were inoculated with 5 μl containing approximately 10⁶ CFU of a culture in early-stationary growth phase. The agarose Pel, Pnl, and Pme plate assays were developed with ruthenium red (0.05%, wt/vol), Peh assay plates with hexadecyl trimethyl ammonium bromide (Cetrimide; 1%, wt/vol), and Cel assay plates with Congo red (0.1%, wt/vol, 20 min) and rinsed one- or twofold with NaCl (1 N). A quantitative determination of extracellular Pel also was performed by measuring the formation of four to five unsaturated products in a spectrophotometer at 235 nm, while Prt activity was quantified by the azocasein method (Smadja et al. 2004).

Soft rot test in tuber.

NaCl-washed, *E. carotovora* subsp. *atroseptica* suspension (cell density $\approx 10^8$ CFU ml⁻¹ in 0.9% NaCl) were obtained

from stationary phase cells, grown in PGA medium. *Solanum tuberosum* cv. Kaptah Vandel tubers were surface sterilized and infected by intramedulla injection (1-cm depth of 10 µl of the above bacterial suspension). The inoculated tubers were incubated in a Minitron incubator (Infors, Massy, France) at 24°C under a relative humidity of 65 ± 2%. The development of the symptoms was evaluated by measuring the weight of the macerated tissue after 12, 24, 48, 72, 96, 192, and 336 h. Strain growth in planta was evaluated at the same time by a dilution and plating technique (on LB agar with appropriate antibiotic) and expressed as CFU g⁻¹ of macerated tissues. For each time and each strain, eight tubers were analyzed to determine the maceration weight and four of those were used to the number of CFU g⁻¹ determination.

HR assays in tobacco plants.

N. tabacum cv. Xanthi XHFD8 and *N. benthamiana* were cultivated for 8 weeks under greenhouse conditions (temperature = 28°C, photoperiod = 16 h). Bacteria were grown in PGA medium at 24°C, washed twice with 0.9% NaCl, and resuspended at 2 × 10⁷, 2 × 10⁸, or 2 × 10⁹ cells ml⁻¹. Tobacco leaves were infiltrated with ≈100 µl of cell suspensions. The margins of the water-soaked infiltrated areas were marked, and the plants were inspected for HR development at 2, 16, 24, and 48 h.

Cloning and sequencing of the *hrc*_{Eca} and *hrpN*_{Eca} genes.

Specific primers amplifying the *hrc*_{Eca} (HrcC1C: 5'-GCTATGCCTCGGTCTCTGAC-3' and HrcC2C: 5'-TGAAATCGCTGACAAACAGG-3') and *hrpN*_{Eca} (HrpN1C: 5'-CACGAGCATTACAGGTTGGTA-3' and HrpN2C: 5'-GATCCGATATGGCGAAAGAA-3') gene have been deduced from the corresponding sequences of *E. carotovora* subsp. *carotovora* SCC1 (Rantakari et al. 2001). Both were used in PCR experiments performed on 1 µg of purified *E. carotovora* subsp. *atroseptica* 6276 chromosomal DNA (94°C for 5 min; 30 cycles consisting in: 30 s at 94°C, 45 s at 60°C, and 45 s at 72°C; followed by a 10-min terminal elongation step). In each case, a single PCR product (516 bp for *hrc*_{Eca} and 274 bp for *hrpN*_{Eca}) was purified from the agarose gel and inserted into the pMOSBlue vector using the pMOSBlue blunt ended cloning kit (Amersham Pharmacia Biotech, Little Chalfont, U.K.), and cloned into *Escherichia coli* XL-1 Blue cells according to standard procedures (Maniatis et al. 1982). White recombinant colonies were selected on LBA plates supplemented with ampicillin (100 µg ml⁻¹), tetracycline (10 µg ml⁻¹), IPTG (24 µg ml⁻¹), and X-Gal (40 µg ml⁻¹). The plasmid content of these colonies was checked by PCR using the two above primers and the nucleotide sequence of the cloned fragments determined from the T7 and U19 priming regions (Genome express, Meylan, France). These sequences were deposited in the GenBank database under GenBank accession number AJ580601 for *hrc*_{Eca} and AJ577998 for *hrpN*_{Eca}. In silico analyses of these sequences were performed with programs available from the Infobiogen Center of Resources. Database searches and analyzes for homologies were performed using the program BLASTP (Altschul et al. 1990). The ClustalW algorithm (Thompson et al. 1994) was used for multiple protein alignments.

RNA extraction.

Total RNAs were extracted using the RNA isolation kit (Roche Diagnostics, Mannheim, Germany) as described by the manufacturer, either from bacteria grown in PGA medium or recovered from tubers. For the latter, macerated tissues were suspended in 3 ml of distilled water and mixed for 1 min exactly. One milliliter was centrifuged at 2,000 × g for 5 min

to pellet plant debris. The supernatant then was centrifuged at 10,000 × g for 5 min to pellet the bacteria and washed two more times with RNase-free ultrapure water. RNA integrity and homogeneity were checked by gel electrophoresis. RNA concentration was determined by measuring the optical density at 260 nm (OD₂₆₀), and purity by measuring the ratio OD₂₆₀/OD₂₈₀.

RT-PCR assays.

RT-PCR experiments were achieved on 10 ng of total RNAs using the one-step RT-PCR procedure (Roche Diagnostics). Primer hybridization temperatures were tested for the RT and PCR steps: the optimal temperatures were 57 and 58°C for *hrc*_{Eca} and *hrpN*_{Eca}, respectively. Absence of contaminating DNA was verified by PCR using the total RNAs preparation as a template (no PCR product was obtained). To calibrate the experiments, an RT-PCR also was performed using primers (R16S1C: 5'-GGGGGTAGAATTCCAGGTGT-3' and R16S2C: 5'-CGGCAGTCTCCTTTGAGTTC-3') that specifically amplify a 495-bp fragment of the *E. carotovora* subsp. *atroseptica* E1 16S ribosomal gene (Fessehaie et al. 2002). A semiquantitative estimation of the concentration of the amplified DNA fragments was realized by Quantity one software (Bio-Rad, Hercules, CA, U.S.A.). Results were expressed as a ratio: *hrc*_{Eca} or *hrpN*_{Eca} transcripts versus 16S transcripts.

Statistical tests.

Unless otherwise specified, all experiments were performed at least three times and the results were reproducible. Differences were considered statistically significant at *P* < 0.05. Mean values and standards errors were indicated. The Fisher least significant difference tests were made with help of the XLSTAT software (Addinsoft, Paris, France).

ACKNOWLEDGMENTS

This study was supported by a grant from Conseil Régional de Haute-Normandie and Comité Nord. We thank J. Brosky (GlaxoSmithKline, USA) for the gift of *Escherichia coli* strain ESS, D. Haas (Université de Lausanne, Switzerland) for the gift of the plasmids pME6000 and pME6863, P. Williams (University of Nottingham, U.K.) for the gift of HSL standards, A. Groboillot for her help in the statistical analysis, and F. Hallak for her technical help.

LITERATURE CITED

- Altschul, S. F., Gish, W., Milles, W., Meyers, E. W., and Lipman, D. J. 1990. Basic local alignment search tool. *J. Mol. Biol.* 215:403-410.
- Atkinson, S., Throup, J. P., Stewart, G. S., and Williams, P. 1999. A hierarchical quorum-sensing system in *Yersinia pseudotuberculosis* is involved in the regulation of motility and clumping. *Mol. Microbiol.* 33:1267-1277.
- Axelrood, P. E., Rella, M., and Schroth, M. N. 1988. Role of antibiosis in competition of *Erwinia* strains in potato infection courts. *Appl. Environ. Microbiol.* 54:1222-1229.
- 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.
- Batchelor, S. E., Cooper, M., Chhabra, S. R., Glover, L. A., Stewart, G. S. A. B., Williams, P., and Prosser, J. I. 1997. Cell density-regulated recovery of starved biofilm populations of ammonia-oxidizing bacteria. *Appl. Environ. Microbiol.* 63:2281-2286.
- Bauer, D. W., Wei, Z.-M., Beer, S. V., and Collmer, A. 1995. *Erwinia chrysanthemi* harpin_{Ech}: an elicitor of the hypersensitive response that contributes to soft rot pathogenesis. *Mol. Plant-Microbe Interact.* 8:484-491.
- Bell, K. S., Avrova, A. O., Holeva, M. C., Cardle, L., Morris, W., de Jong, W., Toth, I. K., Waugh, R., Bryan, G. J., and Birch, P. R. J. 2002. Sample

- sequencing of a selected region of the genome of *Erwinia carotovora* subsp. *atroseptica* reveals candidate phytopathogenicity genes and allows comparison with *Escherichia coli*. *Microbiology* 148:1367-1378.
- Bullock, W. O., Fernandez, J. M., and Short, J. M. 1987. XL1-Blue: a high efficiency plasmid transforming *recA* *Escherichia coli* strain with beta-galactosidase selection. *BioTechniques* 5:376-378.
- Byers, J. T., Lucas, C., Salmond, G. P. C., and Welch, M. 2002. Nondenzy-matic turnover of an *Erwinia carotovora* quorum-sensing signaling molecule. *J. Bacteriol.* 184:1163-1171.
- Carlier, A., Uroz, S., Smadja, B., Fray, R., Latour, X., Dessaux, Y., and Faure, D. 2003. The Ti plasmid of *Agrobacterium tumefaciens* harbors an *attM*-paralogous gene, *aiiB*, also encoding *N*-acyl homoserine lacto-nase activity. *Appl. Environ. Microbiol.* 69:4989-4993.
- Cha, C., Gao, P., Chen, Y.-C., Shaw, P. D., and Farrand, S. K. 1998. Pro-duction of acyl-homoserine lactone quorum-sensing signals gram-nega-tive plant-associated bacteria. *Mol. Plant-Microbe Interact.* 11:1119-1129.
- Chatterjee, A., Cui, Y., Liu, Y., Dumenyo, C. K., and Chatterjee, A. K. 1995. Inactivation of *rsmA* leads to overproduction of extracellular pec-tinases, cellulases, and proteases in *Erwinia carotovora* subsp. *caroto-vora* in the absence of the starvation/cell density-sensing signal, *N*-(3-oxohexanoyl)-L-homoserine lactone. *Appl. Environ. Microbiol.* 61:1959-1967.
- Chilton, M.-D., Currier, T. C., Farrand, S. K., Bendich, A. J., Gordon, M. P., and Nester, E. W. 1974. *Agrobacterium tumefaciens* and PS8 bacte-riophage DNA not detected in crown gall tumors. *Proc. Natl. Acad. Sci. U.S.A.* 71:3672-3676.
- Collmer, A., and Beer, S. V. 1998. *hrp* genes and their function. Pages 139-148 in: *Methods in Microbiology*. Vol. 27. P. Williams, J. Ketley, and G. Salmond, eds. Academic Press, London.
- Cui, Y., Madi, L., Mukherjee, A., Dumenyo, C. K., and Chatterjee, A. K. 1996. The *RsmA*- mutants of *Erwinia carotovora* subsp. *carotovora* strain Ecc71 overexpress *hrpN*Ecc and elicit a hypersensitive reaction-like response in tobacco leaves. *Mol. Plant-Microbe Interact.* 9:565-573.
- Deng, W.-L., Preston, G., Collmer, A., Chang, C.-J., and Huang, H.-C. 1998. Characterization of the *hrpC* and *hrpRS* operons of *Pseudomonas syringae* pathovars *syringae*, *tomato*, and *glycinea* and analysis of the ability of *hrpF*, *hrpG*, *hrcC*, *hrpT*, and *hrpV* mutants to elicit the hyper-sensitive response and disease in plants. *J. Bacteriol.* 180:4523-4531.
- Dong, Y.-H., Xu, J.-L., Li, X.-Z., and Zhang, L.-H. 2000. *AiiA*, an enzyme that inactivates the acylhomoserine lactone quorum-sensing signal and attenuates the virulence of *Erwinia carotovora*. *Proc. Natl. Acad. Sci. U.S.A.* 97:3526-3531.
- Downie, B., Dirk, L. M. A., Hadfield, K. A., Wilkins, T. A., Bennett, A. B., and Bradford, K. J. 1998. A gel diffusion assay for quantification of pectin methyl-esterase activity. *Anal. Biochem.* 264:149-157.
- Eberl, L., Molin, S., and Givskov, M. 1999. Surface motility of *Serratia liquefaciens* MG1. *J. Bacteriol.* 181:1703-1712.
- Fessehaie, A., De Boer, S. H., and Levesque, C. A. 2002. Molecular char-acterization of DNA encoding 16S-23S rRNA intergenic spacer regions and 16S rRNA of pectolytic *Erwinia* species. *Can. J. Microbiol.* 48:387-398.
- Gardan, L., Gouy, C., Christen, R., and Samson, R. 2003. Elevation of three subspecies of *Pectobacterium carotovorum* to species level: *Pecto-bacterium atrosepticum* sp. nov., *Pectobacterium betavascularum* sp. nov. and *Pectobacterium wasabiae* sp. nov. *Int. J. Syst. Evol. Microbiol.* 53:381-391.
- Ham, J. H., Cui, Y., Alfano, J. R., Rodriguez-Palenzuela, P., Rojas, C. M., Chatterjee, A. K., and Collmer, A. 2004. Analysis of *Erwinia chrysanthemi* EC16 *pelE::uidA*, *pelL::uidA*, *hrpN::uidA* mutants reveals strain-specific atypical regulation of the Hrp type III secretion system. *Mol. Plant-Microbe Interact.* 17:184-194.
- 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 regu-lates motility and the expression of multiple virulence determinants. *Mol. Microbiol.* 28:705-717.
- Hauben, L., Moore, E. R. B., Vauterin, L., Steenackers, M., Mergaert, J., Verdonck, L., and Swings, J. 1998. Phylogenetic position of phytopa-thogens within the Enterobacteriaceae. *Syst. Appl. Microbiol.* 21:384-397.
- 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., Winslow, M. K., Salmond, G. P. C., Stewart, G. S. A. B., and Williams, P. 1993. The *Lux* autoinducer regulates the production of exoenzyme virulence determi-nants in *Erwinia carotovora* and *Pseudomonas aeruginosa*. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:2477-2482.
- Josenhans, C., and Suerbaum, S. 2002. The role of motility as a virulence factor in bacteria. *Int. J. Med. Microbiol.* 291:605-614.
- Kim, J. F., Wei, Z. M., and Beer, S. V. 1997. The *hrpA* and *hrpC* operons of *Erwinia amylovora* encode components of a type III pathway that se-cretes harpin. *J. Bacteriol.* 179:1890-1897.
- Kim, J. F., Ham, J. H., Bauer, D. W., Collmer, A., and Beer, S. V. 1998. The *hrpC* and *hrpN* operons of *Erwinia chrysanthemi* EC16 are flanked by *plcA* and homologs of hemolysin/adhesin genes and accompanying activator/transporter genes. *Mol. Plant-Microbe Interact.* 11:563-567.
- Köhler, T., Curty, L. K., Barja, F., Van Delden, C., and Pechère, J.-C. 2000. Swarming of *Pseudomonas aeruginosa* is dependent on cell-to-cell sig-naling and requires flagella and pili. *J. Bacteriol.* 182:5990-5596.
- Leadbetter, J. R., and Greenberg, E. P. 2000. Metabolism of acyl-homoser-ine lactone quorum-sensing signals by *Variovorax paradoxus*. *J. Bacteriol.* 182:6921-6926.
- Lee, S. J., Park, S.-Y., Lee, J.-J., Yum, D.-Y., Koo, B.-T., and Lee, J.-K. 2002. Genes encoding the *N*-acyl homoserine lactone-degrading en-zyme are widespread in many subspecies of *Bacillus thuringiensis*. *Appl. Environ. Microbiol.* 68:3919-3924.
- Liao, C.-H. 1989. Analysis of pectate lyases produced by soft rot bacteria associated with spoilage of vegetables. *Appl. Environ. Microbiol.* 55:1677-1683.
- Mäe, A., Montesano, M., Koiv, V., and Palva, E. T. 2001. Transgenic plants producing the bacterial pheromone *N*-acyl-homoserine lactone exhibit enhanced resistance to the bacterial phytopathogen *Erwinia carotovora*. *Mol. Plant-Microbe Interact.* 14:1035-1042.
- Maniatis, T., Fritsch, E. F. and Sambrook, J. 1982. *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, U.S.A.
- Marits, R., Koiv, V., Laasik, E., and Mäe, A. 1999. Isolation of an extracel-lular protease gene of *Erwinia carotovora* subsp. *carotovora* strain SCC3193 by transposon mutagenesis and the role of protease in phyto-pathogenicity. *Microbiology* 145:1959-1966.
- Maurhofer, M., Reimann, C., Schmidli-Sacherer, P., Heeb, S., Haas, D., and Défago, G. 1998. Salicylic acid biosynthetic genes expressed in *Pseudomonas fluorescens* strain P3 improve the induction of systemic resistance against tobacco in *Tobacco necrosis virus*. *Phytopathology* 88:678-684.
- 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 *Chromobacte-rium violaceum*: exploitation of violacein production and inhibition for the detection of *N*-acylhomoserine lactones. *Microbiology* 143:3703-3711.
- McEvoy, J. L., Murata, H., and Chatterjee, A. K. 1990. Molecular cloning and characterization of an *Erwinia carotovora* subsp. *carotovora* pectin lyase gene that responds to DNA-damaging agents. *J. Bacteriol.* 172:3284-3289.
- Moens, S., and Vanderleyden, J. 1996. Functions of bacterial flagella. *CRC Crit. Rev. Microbiol.* 22:67-100.
- Molina, L., Constantinescu, F., Michel, L., Reimann, C., Duffy, B., and Défago, G. 2003. Degradation of pathogen quorum-sensing molecules by soil bacteria: a preventive and curative biological control mecha-nism. *FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Ecol.* 45:71-81.
- Mukherjee, A., Cui, Y., Liu, Y., and Chatterjee, A. K. 1997. Molecular characterization and expression of the *Erwinia carotovora hrpN*_{Ecc} gene, which encodes an elicitor of the hypersensitive reaction. *Mol. Plant-Microbe Interact.* 10:462-471.
- Mulholland, V., Hinton, J. C. D., Sidebotham, J., Toth, I. K., Hyman, L. J., Pérombelon, M. C. M., Reeves, P. J., and Salmond, G. P. C. 1993. A pleiotropic reduced virulence (*Rvi*⁻) mutant of *Erwinia carotovora* sub-species *atroseptica* is defective in flagella assembly proteins that are conserved in plant and animal bacterial pathogens. *Mol. Microbiol.* 9:343-356.
- Nasser, W., Bouillant M. L., Salmond, G. P. C., and Reverchon, S. 1998. Characterization of the *Erwinia chrysanthemi expl-expR* locus directing the synthesis of two *N*-acyl-homoserine lactone signal molecules. *Mol. Microbiol.* 29:1391-1405.
- Pérombelon, M. C. M. 2002. Potato diseases caused by soft rot erwinias: an overview of pathogenesis. *Plant Pathol.* 51:1-12.
- Pérombelon, M. C. M., and Salmond, G. P. C. 1995. Bacterial soft rots. Pages 1-20 in: *Pathogenesis and Host Specificity in Plant Diseases*. I. U. S. Singh, R. P. Singh, and K. Kohmoto, eds. Pergamon Press, Oxford.
- Piper, K. R., Beck von Bodman, S., and Farrand, S. K. 1993. Conjugation factor of *Agrobacterium tumefaciens* regulates Ti plasmid transfer by autoinduction. *Nature* 362:448-450.
- Pirhonen, M., Flego, D., Heikinheimo, R., and Palva, E. T. 1993. A small diffusible signal molecule is responsible for the global control of viru-lence and exoenzyme production in plant pathogen *Erwinia carotovora*. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:2467-2476.
- Priou, S., and Jouan, B. 1996. Les maladies provoquées par les bactéries pathogènes du genre *Erwinia*. Pages 260-265 in: *La pomme de terre*. P. Rousselle, Y. Robert, and J. C. Crosnier, eds. INRA Editions, Paris.
- Rantakari, A., Virtaharju, O., Vähämäki, S., Taira, S., Palva, E. T., Saari-lahti, H. T., and Romantschuk, M. 2001. Type III secretion contrib-

- utes to the pathogenesis of the soft-rot pathogen *Erwinia carotovora*: partial characterization of the *hrp* gene cluster. *Mol. Plant-Microbe Interact.* 14:962-968.
- Reimmann, C., Ginot, N., Michel, L., Keel, C., Michaux, P., Krishnapillai, V., Zala, M., Heurlier, K., Triandafyllu, K., Harms, H., Défago, G., and Haas, D. 2002. Genetically programmed autoinducer destruction reduces virulence gene expression and swarming motility in *Pseudomonas aeruginosa* PAO1. *Microbiology* 148:923-932.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, U.S.A.
- Scott, R. I., Chard, J. M., Hocart, M. J., Lennard, J. H., and Graham, D. C. 1996. Penetration of potato tuber lenticels by bacteria in relation to biological control of blackleg disease. *Potato Res.* 39:333-344.
- Shaw, P. D., Ping, G., Daly, S. L., Cha, C., Cronan, J. E., Jr., Rinehart, K. L., and Farrand, S. K. 1997. Detecting and characterizing *N*-acyl-homoserine lactone signal molecules by thin-layer chromatography. *Proc. Natl. Acad. Sci. U.S.A.* 94:6036-6041.
- Smadja, B., Latour, X., Trigui, S., Burini, J. F., Chevalier, S., and Orange, N. Thermodependence of growth and enzymatic activities implicated in pathogenicity of two *Erwinia carotovora* subspecies (*Pectobacterium* spp.). *Can. J. Microbiol.* 50:19-27.
- Thompson, J. D., Higgins, D. G., and Gibson, T. J. 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighing, position-specific gap penalties and weight matrix choice. *Nucleic Acids. Res.* 22:4673-4680.
- Thorne, S. H., and Williams, H. D. 1999. Cell density-dependent starvation survival of *Rhizobium leguminosarum* bv. *phaseoli*: identification of the role of an *N*-acyl homoserine lactone in adaptation to stationary-phase survival. *J. Bacteriol.* 181:981-990.
- Toth, I. K., Bell, K. S., Holeva, M. C., and Birch, P. R. J. 2003. Soft rot *erwiniae*: from genes to genomes. *Mol. Plant Pathol.* 4:17-30.
- Toth, I. K., Thorpe, C. J., Bentley, S. D., Mulholland, V., Hyman, L. J., Pérombelon, M. C. M., and Salmond, G. P. C. 1999. Mutation in a gene required for lipopolysaccharide and enterobacterial common antigen biosynthesis affects virulence in the plant pathogen *Erwinia carotovora* subsp. *atroseptica*. *Mol. Plant-Microbe Interact.* 12:499-507.
- Uroz, S., D'Angelo-Picard, C., Carlier, A., Elasm, M., Sicot, C., Petit, A., Oger, P., Faure, D., and Dessaux, Y. 2003. Novel bacteria degrading *N*-acylhomoserine lactones and their use as quenchers of quorum-sensing-regulated functions of plant-pathogenic bacteria. *Microbiology* 149:1981-1989.
- Wei, Z. M., Laby, R. J., Zumoff, C. H., Bauer, D. W., He, S. Y., Collmer, A., and Beer, S. V. 1992. Harpin, elicitor of the hypersensitive response produced by the plant pathogen *Erwinia amylovora*. *Science* 257:85-88.
- Whitehead, N. A., Barnard, A. M. L., Slater, H., Simpson, N. J. L., and Salmond, G. P. C. 2001. Quorum-sensing in Gram-negative bacteria. *FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Rev.* 25:365-404.
- Whitehead, N. A., Byers, J. T., Commander, P., Corbett, M. J., Coulthurst, S. J., Everson, L., Harris, A. K. P., Pemberton, C. L., Simpson, N. J. L., Slater, H., Smith, D. S., Welch, M., Williamson, N., and Salmond, G. P. C. 2002. The regulation of virulence in phytopathogenic *Erwinia* species: quorum sensing, antibiotics and ecological considerations. *Antonie van Leeuwenhoek* 81:223-231.

AUTHOR-RECOMMENDED INTERNET RESOURCE

Infobiogen Center of Resources: www.infobiogen.fr