The Role of Type III Secretion System in *Erwinia carotovora* subsp. *atroseptica* Virulence

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Abstract

A *hrpJ* mutant of *Erwinia carotovora* subsp. *atroseptica* (*Eca*) was constructed and the effects of this mutation on induction of the hypersensitivity response and virulence of *Eca* were studied. The *hrpJ* mutant does not induce the HR in non-host plant *Vicia faba* (*V. faba*) and demonstrates cultivar-dependent increase or decrease of virulence on potato plants.

Keywords: *Erwinia carotovora* subsp. *atroseptica*; potato; type III secretion system; virulence; hypersensitive response

INTRODUCTION

Pectolytic *Erwinia* (*E. chrysanthemi* and *E. carotovora*) are probably unique among phytopathogens in that they are capable, at least in experimental conditions, to cause disease in a broad range of plants. On the other hand, these bacteria are hardly able to overcome surface defence barriers of a healthy plant and are thought to infect plant tissues only through the sites of mechanical damage. Once the first defence barrier is broken *Erwinia* can multiply in plant tissues due to massive production of extracellular depolymerases (pectate lyases, polygalacturonases, proteases and cellulases).

More specialised pathogens, such as strains of *Pseudomonas syringae, Xanthomonas campestris* and *Ralstonia solanacearum*, are much better adapted to a particular host, but this inevitably results in host range of these strains being restricted to a relatively narrow range of "compatible" plants. The contact of a narrow host range pathogen with resistant or "incompatible" plant results in rapid localised death of plant cells surrounding the site of pathogen infection. This phenomenon called the "hypersensitivity response" (HR) blocks further spread of the pathogen within plant tissues and appears to be a universal resistance mechanism that plants utilise against any microorganism they can recognise as pathogenic. The molecular machinery behind compatible and incompatible interactions has been actively studied during the last 15 years and a lot of information is already available. It appears that pathogenic bacteria able to cause HR have a large (25–30 kb) cluster of genes absolutely required for elicitation of the HR and in many cases for pathogenicity, therefore this cluster was named *hrp* for *h*ypersensitivity *r*esponse and *p*athogenicity (LINDGREN *et al*. 1986). The *hrp* cluster codes for several secreted proteins (harpins and Avr proteins) as well as for the type III secretion apparatus required for their delivery to special locations within plant cells – usually cell wall/membrane for harpins and plant cell cytoplasm or nucleus for Avr proteins (see LINDGREN 1997 for a review). Avr proteins received their name because their presence can induce the HR in a host if the plant has the corresponding resistance (*R*) gene. This gene-for-gene mechanism of interaction, originally suggested by FLOR (1956), is what restricts the host range of many pathogens. Avr proteins probably increase the virulence of a pathogen on certain hosts, but if the plant has a matching *R* gene, HR is induced leading to incompatible interaction.

Little attention was paid to the *hrp* system in pectolytic *Erwinia* until recently, because of the above mentioned extracellular hydrolases, which their corresponding secretion systems (type II and type I) and regulatory networks were thought to be the most

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important virulence factors in these bacteria. It was shown that *E. chrysanthemi,* although having the *hrp* genes, normally does not induce the HR due to very high activity of macerases masking typical HR symptoms and a mutant with inactivated type III secretion system has only minor reduction of virulence (BAUER *et al*. 1994). A recent study of the *hrp* system in *Erwina carotovora* subsp. *carotovora* also did not show clear decrease of virulence of a *hrcC* mutant on *Arabidopsis* plants, although some delay in symptom development was detected (RANTAKARI *et al*. 2001).

To further investigate the role of type III secretion system in pectolytic *Erwinia* we have chosen another subspecies (*atroseptica*) of *Erwinia carotovora* as probably the most specialised of the soft rot erwinias. The host range of this subspecies is thought to be restricted to potato plants (BAIN & PEROMBELON 1988). In addition to causing soft rot of potato tubers (which is typical for pectolytic erwinias), *E. carotovora* subsp. *atroseptica* can cause a blackleg of potato stems. Therefore this "narrow host range" representative of pectolytic *Erwinia* looked like a good candidate for studying the role of type III secretion system in *Erwinia* virulence.

MATERIALS AND METHODS

Bacterial strains, plasmids and media. *Erwinia carotovora* subsp. *atroseptica* strain JN42 (*Eca*) is a rif^R r^- derivative of a wild type *Eca* strain 3–2. Both strains are from the collection of the Microbiology Department of Belarus State University. The *Escherichia coli* strain XL-1 Blue (BULLOCK *et al*. 1987) was primarily used for plasmid construction and *E. coli* strain BW19851 (METCALF *et al*. 1994) was used for conjugational transfer of suicide vector pJQ200mp18 into *Eca*. Cloning vector pUC18 (NORRANDER *et al*. 1983) and suicide vector pJQ200mp18 (QUANDT & HYNES 1993) were used throughout this work. *Eca* and *E. coli* were grown in LB broth at 28° and 37°C, respectively. Antibiotics were used at the following concentrations (μg/ml): ampicillin 100; gentamycin 10; rifampicin 25; streptomycin 50.

DNA manipulations. Manipulations with DNA and PCR were performed according to standard protocols (AUSUBEL *et al*. 1992). DNA sequencing was performed on an automated DNA sequencer ALFexpress II (Amersham Pharmacia Biotech.). Restriction endonucleases, DNA ligase, DNA extraction kit and DNA sequencing kit were purchased from MBI Fermentas. Transformation of *E. coli* was performed by a standard calcium chloride method (AUSUBEL *et al*. 1992).

Virulence assays. Potato *Solanarum tuberosum* (*S. tuberosum)* and broad bean *V. faba* plants were grown in a greenhouse at 25°C with a photoperiod of 16 h for two weeks prior to inoculation. Bacteria prior inoculation were grown overnight on solid LB plates, washed off with 0.85% NaCl solution, centrifuged briefly and resuspended in the same solution, after which the OD590 values were adjusted to give the required cell densities (3×10^8 cells/ml). Upper parts of two-week-old potato stems were inoculated with a Hamilton syringe in three places with 10 μl of bacterial suspension per each injection. The plants were monitored daily over a 4-day period for symptom development. The maximal symptom development was scored for each stem. For HR assays, 40 μl of bacterial suspensions (10^8 cells/ml) were infiltrated per bean leaf with a Hamilton syringe and the results were scored the next day.

Construction of the hrpJ mutant. To construct the *hrpJ* mutation in *Eca* a fragment of chromosomal DNA was amplified by PCR using the following primers: HRPL1 (5′CCAGAGCTCGCDATNCCRAANACCCCANGT3′) and HRPJ1 (5′CACTCTAGATTGGCGCTCAGGCCAATCAG3′). The amplified DNA was cut with *Sac*I and *Xba*I, gel purified, and cloned into pUC18. The omega interposon (PRENTKI & KRISCH 1984) coding for streptomycin/spectinomycin resistance was introduced into the cloned *hrp* fragment in *Ava*I site within the *hrpJ* gene (after Klenow treatment). The resulting *hrpJ*::ω piece was subcloned with *Sac*I and *Xba*I into the same sites of pJQ200mp18 and transformed into *E. coli* strain BW19851. Then the pJQ200mp18-*hrp*::ω construct was transferred from *E. coli* BW19851 to *Eca* by conjugation. The *Eca* mutants with hrp::ω insertion were selected in 5% sucrose media with streptomycin, according to (QUANDT & HYNES 1993). The resulting *hrpJ* mutant (JN501strain) was verified by PCR.

RESULTS

Cloning and sequencing of the Eca hrpJ gene. During unrelated project we have previously sequenced a fragment of *Eca* 3-2 DNA that contained the junction of *hrpJ* and *hrcV* genes (NIKOLAICHIK unpubl.). This allowed us to design a pair of primers for PCR amplification of a DNA fragment sufficiently long to be used for marker exchange mutagenesis. The primers HRPJ1 (complementary to previously determined *hrpJ* sequence) and HRPL1 (made according to reverse translation of the most conservative part of available HrpL sequences from other bacteria) allowed us to

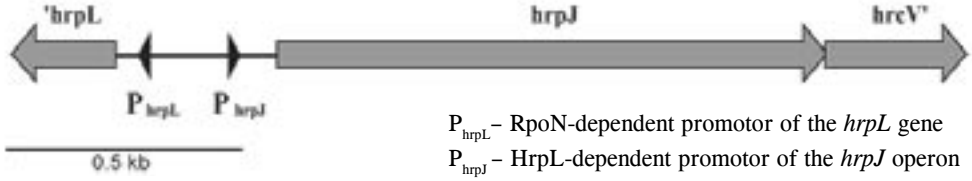

Figure 1. DNA fragment (2079bp) of *Eca* type III secretion system, containing *'hrpL*, *hrpJ* and *hrcV'* genes

amplify 1.4 kb fragment of chromosomal DNA covering the *hrpJ* gene and the very beginning of the *hrpL* gene. This PCR fragment was cloned in pUC18 and sequenced (EMBL accession number: AJ496800). This PCR fragment was cloned in pUC18 and sequenced (EMBL accession number: AJ496800). The sequenced fragment contains one complete and two partial open reading frames coding for proteins with high homologies to HrpL, HrpJ and HrcV proteins from *Erwinia amylovora* (Figure 1). Open reading frames coding for HrpJ and HrcV overlap by four nucleotides suggesting an operon organisation of these two genes. Divergently transcribed *hrpL* and *hrpJ* genes are separated by 328 bases and two putative promoters could be identified within this region. There is a sequence (TGGCACAAGGCTTGCT) perfectly matching the σ^{54}-dependent promoter consensus sequence located 45 bases upstream of start-codon of the *hrpL* gene. Located 99 bp upstream of *hrpJ* is a sequence GGAACC-$(N)_{15}$-CCACACA with good resemblance to HrpL-dependent promoter. The presence of these two promoters suggests the possibility of cascade regulation of *hrp* genes analogous to the regulatory network in *P. syringae*.

hrpJ mutant is unable to induce the hypersensitive response on leaves of a non-host plant. To elucidate the role of type III secretion system in *Eca* virulence the *hrpJ* mutant was constructed through the marker exchange mutagenesis as described in materials and methods. As the induction of HR is a typical sign of a functional type III secretion system, both the wild type and the *hrpJ* mutant strains were tested for the ability to elicit the hypersensitive response by inoculation into the leaves of *V. faba* (a non-host plant). While the wild type cells gave clear HR within 16 h after inoculation, no HR-like symptoms was observed with the *hrpJ* mutant strain (Figure 2). It should be noted, however, that when higher inoculum doses (10^9 cells/ml) were used, the *hrpJ* mutant was able to cause HR, although the development of symptoms was somewhat delayed.

Inactivation of the hrpJ gene results in cultivar-specific alteration of Eca virulence on potato plants. As significantly reduced ability of the *hrpJ* mutant to induce HR symptoms is a good indication of inactivation of the type III secretion system (or at least severe alteration of its functionality) we continued with tests on potato stems and tubers to assay the role of this secretion system in virulence on the host plants. On potato stems we have observed no difference in virulence between the JN501 and the wild type strains when inoculum doses were high (10^9 cells/ml). However, when inoculum doses were reduced so that only about 50% of stems inoculated with the wild type strain were developing typical blackleg symptoms, the difference between two strains was significant. Depending on potato cultivar used for stem inoculations, the *hrpJ* mutant demonstrated either increased or reduced virulence (Figure 3).

The JN501 strain appeared to have significantly increased virulence on cultivar Yavar – much more stems have developed the complete blackleg symptoms and stem damage by the mutant was already visible 16 h after inoculation whereas for the wild type strain at least 40 h were required for visible symptoms at

Figure 2. The induction of the hypersensitivity reaction by *Eca* strains on *V. faba*

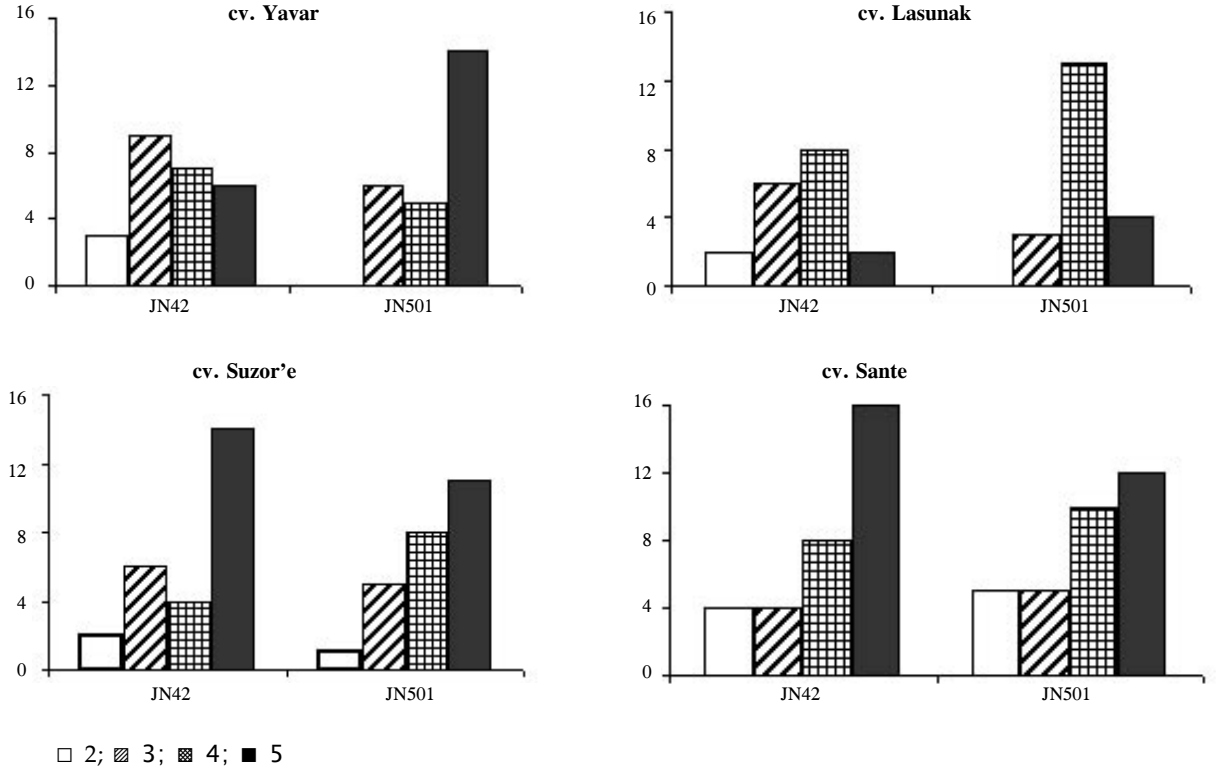

Fig. 3. Virulence of JN501 and JN42 *Eca* strains on different cultivars of *S. tuberosum*. Disease symptom development was scored according to the following scale: 0 – no visible changes; 1 – light browning around the site of inoculation; 2 – slight rotting of tissues (1–2 mm deep) around the site of inoculation; 3 – local rotting of internal stem tissues; 4 – medium rotting of internal and (partially) external stem tissues, spreading slightly from the site of inoculation, external rotted tissues may turn black; 5 – complete blackleg symptoms: the whole interleaf interval is black, dry, shrunk in diameter. The scale on the left represents the number of plants affected by the corresponding symptom

this inoculum dose. The virulence of the mutant was also higher than that of the wild type strain on cv. Lasunak, and slightly lower on cv. Suzor'e and cv. Sante.

DISCUSSION

While this work was in progress, a paper was published demonstrating the presence of type III secretion system in *Erwinia carotovora* subsp. *atroseptica* (BELL *et al.* 2002). However, no finished sequences were reported in this work and the role of the type III secretion system in *Eca* was not studied as well. Here we report the complete sequence of one gene from this system, *hrpJ*, and assay its role in *Eca* virulence.

The component of type III secretion system coded by the *hrpJ* gene is not very conserved and its function is not clear. However, this gene is the first gene in the operon. The next gene, *hrcV*, codes for a very conserved component of type III secretion apparatus and it is known that its knockout blocks type III secretion (HUANG *et al.* 1993; WEI & BEER 1993). As the *hrcV* and *hrpJ* genes overlap and there is a reasonable match to a HrpL-dependent promoter located upstream of *hrpJ* with no additional promoters visible in front of *hrcV*, a polar insertion of omega interposon within the *hrpJ* gene should inactivate the *hrcV* gene as well, or at least significantly reduce its transcription. The inactivation of the whole type III secretion system in the *hrpJ* mutant is supported by its failure to induce the HR symptoms on *V. faba* leaves at normal inoculum doses. The residual necrosis of leaf tissue resembling the HR which is caused by the mutant at high inoculum doses may be due to the activity of pectate lyases because an injection of a pure preparation of at least one of them into tobacco leaves has been reported to cause the HR-like necrosis (SHEVCHIK *et al.* 1998).

The inactivation of type III secretion system has also affected the virulence of *Eca* on the host plant. Although there was no clear effect of the *hrpJ* mutation on tuber maceration, stem inoculations demonstrated marked

difference between the wild type and mutant strains. The *hrpJ* mutant appeared to be more virulent on stems of some potato cultivars while being less virulent or indistinguishable from wild type on others.

Our results confirm previous reports that the role of type III secretion system in virulence of pectolytic *Erwinia* is probably less important than that of extracellular hydrolases produced by these bacteria. Nevertheless, we have observed that the type III secretion system in a pectolytic *Erwinia* strain is responsible for cultivar-dependent differencies in virulence. Hence, even if the type III secretion system and its substrates are not the major virulence factor in this bacterium, as in narrow host range pathogens, the *hrp* system can still be utilised by bacteria for fine tuning the invasion strategy for a particular host plant.

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