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hrp Genes of *Pseudomonas solanacearum* are Homologous to Pathogenicity Determinants of Animal Pathogenic Bacteria and are Conserved Among Plant Pathogenic Bacteria

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The majority of bacterial plant diseases are caused by members of three bacterial genera, *Pseudomonas*, *Xanthomonas*, and *Erwinia*. The identification and characterization of mutants that have lost the abilities to provoke disease symptoms on a compatible host and to induce a defensive hypersensitive reaction (HR) on an incompatible host have led to the discovery of clusters of *hrp* genes (hypersensitive reaction and pathogenicity) in phytopathogenic bacteria from each of these genera. Here, we report that predicted protein sequences of three *hrp* genes from *Pseudomonas solanacearum* show remarkable sequence similarity to key virulence determinants of animal pathogenic bacteria of the genus *Yersinia*. We also demonstrate DNA homologies between *P. solanacearum hrp* genes and *hrp* gene clusters of *P. syringae* pv. *phaseolicola*, *Xanthomonas campestris* pv. *campestris*, and *Erwinia amylovora*. By comparing the role of the *Yersinia* determinants in the control of the extracellular production of proteins required for pathogenicity, we propose that *hrp* genes code for an export system that might be conserved among many diverse bacterial pathogens of plants and animals but that is distinct from the general export pathway.

Pseudomonas solanacearum (Smith) Smith is the causal agent of bacterial wilt of Solanaceous crops and many other plants (Buddenhagen and Kelman 1964). Most mutants of the strain GMI1000 of *P. solanacearum* that have lost the ability to induce disease symptoms on tomato (compatible plant) and an HR on tobacco (incompatible plant) (Boucher *et al.* 1985) map to a large cluster of *hrp* genes (Boucher *et al.* 1987). An additional *hrp* locus, which is smaller in size, has been described in strain K60 of *P. solanacearum* (Huang *et al.* 1990). The large *hrp* gene cluster of strain GMI1000 has been characterized by the aid of localized Tn*5*-B20 mutagenesis (Arlat *et al.* 1992). This enabled the left- and right-hand limits of the cluster to be determined and six potential transcription units to be defined. DNA sequence analysis of the cluster is currently underway.

It has been shown that *hrp* genes are functionally conserved between related bacteria (Willis *et al.* 1991). For example, there is conservation of *hrp* gene function within pathovars of *P. syringae* van Hall (Lindgren *et al.* 1988), pathovars of *Xanthomonas campestris* (Pammel) Dowson (Bonas *et al.* 1991; Arlat *et al.* 1991), strains of *P. solanacearum* (Boucher *et al.* 1988), and probably even between two different bacterial species, *P. solanacearum* and *X. campestris* (Arlat *et al.* 1991). Homology has been detected at the level of DNA hybridization between various *Erwinia* species and between *Erwinia amylovora* (Burrill) Winslow *et al.* and *P. syringae* (Laby and Beer 1990). This widespread occurrence and conservation of *hrp* genes among phytopathogenic bacteria implies that many different bacterial plant diseases involve common mechanisms. Moreover, *hrp* genes may code for basic pathogenicity functions, superimposed on which are factors that contribute to disease expression but vary from one bacterium to another.

Despite the widespread discovery of *hrp* genes, very little is known about the mechanisms by which *hrp* gene products control and determine the outcome of plant-pathogen interactions. In this paper, we demonstrate that there is remarkable sequence similarity between Hrp proteins of *P. solanacearum* and virulence determinants of *Yersinia enterocolitica* (Schleifstein and Coleman) Frederiksen and *Y. pestis* (Lehmann and Neumann) Van Loghem (Michiels *et al.* 1991; Plano *et al.* 1991), which are human pathogenic bacteria for which substantially more information is available about the molecular basis of disease (Cornelis *et al.* 1989; Smirnov 1990). That these *Yersinia* virulence determinants are involved in the secretion of proteins required for pathogenicity suggests an analagous role for *hrp* genes and represents a major breakthrough in the understanding of the mechanisms of bacterial plant pathology.

To find out whether *hrp* gene products of other bacteria are likely to show similar sequence similarities, we performed Southern blot hybridizations between the three *P. solanacearum hrp* genes featured in this paper and *hrp* genes of other bacterial species. These experiments showed that there is DNA homology between these particular *P. solanacearum hrp* genes and *hrp* gene clusters of *P. s.* pv. *phaseolicola* (Burkholder) Young *et al.*, *X. c.* pv. *campestris* (Pammel) Dowson, and *E. amylovora*; the significance of this homology is demonstrated in an accompanying paper (Fenselau *et al.* 1992), which presents sequence similarities between virulence determinants of *Yersinia* and predicted Hrp proteins of *X. c.* pv. *vesicatoria* (Doidge) Dye.

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Nucleotide and/or amino acid sequence data were submitted to GenBank as accession numbers M99631, M99632, and M99633.

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MATERIALS AND METHODS

DNA manipulations. DNA purification, restriction enzyme analysis, and DNA ligations were performed by use of standard techniques.

DNA sequencing. Deletion derivatives of appropriate plasmids were generated (Henikoff 1984). Selected deletions were chosen, and single-stranded DNA was purified from strains after infection with the phage M13K07; techniques described by Stratagene specifically designed for Bluescript vectors (DNA Sequencing Instruction Manual, Stratagene, Inc., La Jolla, CA) were used. DNA sequencing reactions were performed by the dideoxy-termination method (Sanger *et al.* 1977), with 5′([^{35}S]thio)deoxyadenosine triphosphate (Du Pont, NEN Research Products; Du Pont, Wilmington, DE) as the radioactive label (Biggin *et al.* 1983); the same Stratagene protocols were used. The complete nucleotide sequence was analyzed by using computer programs in version 7.0 of the GCG Sequence Analysis Software Package (Genetics Computer Group, Inc., Madison, WI) (Devereux *et al.* 1984). In particular, the protein sequence alignments were produced with the program PILEUP from this package. The protein sequences were analyzed for potential N-terminal signal sequences, and membrane-spanning domains were predicted with release 5.0 of the PC/Gene Programme, Department of Medical Biochemistry, University of Geneva, Switzerland.

Southern blot hybridizations. DNA probes were radioactively labeled with ^{32}P by using random oligonucleotide primers. Hybridizations were performed at 37° C (in 50% formamide, 6× SSC [1× SSC = 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0], 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, 0.2% Ficoll, 50 μg/ml of salmon sperm DNA), and filters were washed at room temperature (in 2× SSC, 0.1% sodium dodecyl sulfate [SDS] followed by 0.1× SSC, 0.1% SDS).

RESULTS AND DISCUSSION

DNA sequence analysis of the 18 kilobases (kb) on the left in the large *hrp* gene cluster of strain GMI1000 of *P. solanacearum* revealed 18 open reading frames (ORFs) with high protein coding probabilities (unpublished data). Figure 1 shows the relative positions of three ORFs (*hrpA*, *hrpI*, and *hrpO*) for which the predicted protein sequences show similarity to deduced sequences of *Yersinia* virulence proteins. The mutations indicated within *hrpA*, *hrpI*, and *hrpO* all result in an Hrp$^-$ phenotype that is not due, at least for mutations in *hrpA* and *hrpO*, to the interruption of downstream *hrp* gene expression.

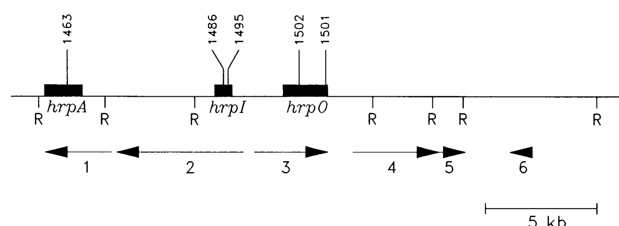

Fig. 1. Location of *hrpA*, *hrpI*, and *hrpO* within the large *hrp* gene cluster of *Pseudomonas solanacearum* GMI1000. Relevant Tn*5*-B20 insertions in which positions have been sequenced are indicated by vertical lines and labeled with the name of the corresponding mutant strain. Arrows below the map correspond to putative transcription units (Arlat *et al.* 1992). R = *Eco*RI.

Homology of proteins encoded with the *hrp* genes to proteins of *Yersinia*. The protein alignments are shown in Figure 2. HrpA of *P. solanacearum* (predicted to contain 568 amino acids [aa] and have a relative molecular mass [M_r] of 60,439 Da) had sequence similarity with YscC of *Y. enterocolitica* W22703 (Michiels *et al.* 1991) as well as with PulD of *Klebsiella pneumoniae* (Schroeter) Trevisan (d'Enfert *et al.* 1989) and the gene IV protein (pIV) of the filamentous bacteriophage I2-2 (EMBL accession no. X14336) (Fig. 2A). The levels of sequence identity between HrpA and each protein are 34.1% with YscC over 683 aa; 22.5% with PulD over 691 aa; and 24.1% with pIV over 582 aa. Each given length represents the total alignment length, including gaps. This conservation of sequence between HrpA and YscC is obvious throughout the proteins, although it is best in the C-terminal domains because it is also between the four proteins.

There is 35.4% identity over 237 aa between HrpI of *P. solanacearum* (predicted to be a preprotein of 269 aa with a M_r of 28,973 Da) and YscJ of *Y. enterocolitica* W22703 (Michiels *et al.* 1991) (Fig. 2B). The sequence similarity extends over practically the entire length of each protein. Both are predicted to be lipoproteins with a lipoprotein-specific signal sequence; the potential signal peptidase II cleavage site of HrpI agrees exactly with the consensus of Leu-(Ala/Ser)-(Gly/Ala) Cys (Hayashi and Wu 1990). Both are also predicted to have a hydrophobic C-terminal domain followed by positively charged residues, a characteristic of integral membrane proteins (Boyd and Beckwith 1990).

HrpO of *P. solanacearum* (predicted to be a protein of 690 aa with M_r of 73,990 Da) and LcrD of *Y. pestis* KIM (Plano *et al.* 1991) align over the entire length of each protein with considerable sequence similarity: 43.8% identity over 720 aa (including gaps) (Fig. 2C). This is especially pronounced in the hydrophobic N-terminal regions and is reflected in the relative positioning of potential membrane-spanning domains and the occurrence of positively charged amino acids between these domains. The deduced sequence of HrpO suggests the presence of nine potential membrane-spanning domains, all located in the N terminal and central parts of the protein. Considerable sequence similarity (63.6% identity over 143 aa) was also found between the N-terminal region of HrpO and the predicted protein sequence of a partial ORF representing the 5′ end of the *lcrD* gene of *Y. enterocolitica* 0:3 (Viitanen *et al.* 1990) (data not shown). Additional similarity was found with a potential protein of *Shigella flexneri* Castellani and Chalmers, another animal pathogen. This homology (32% identity over 189 aa) is between the C terminus of HrpO and the C terminus of the potential protein encoded upstream of the *spa15* gene (EMBL accession no. M81458), which is located within a pathogenicity locus (data not shown). These two last homologies were detected with the TFASTA program.

Mutations in the transcriptional units to which *yscC*, *yscJ*, and *lcrD* map completely inhibit the translocation of extracellular virulence determinants termed Yops

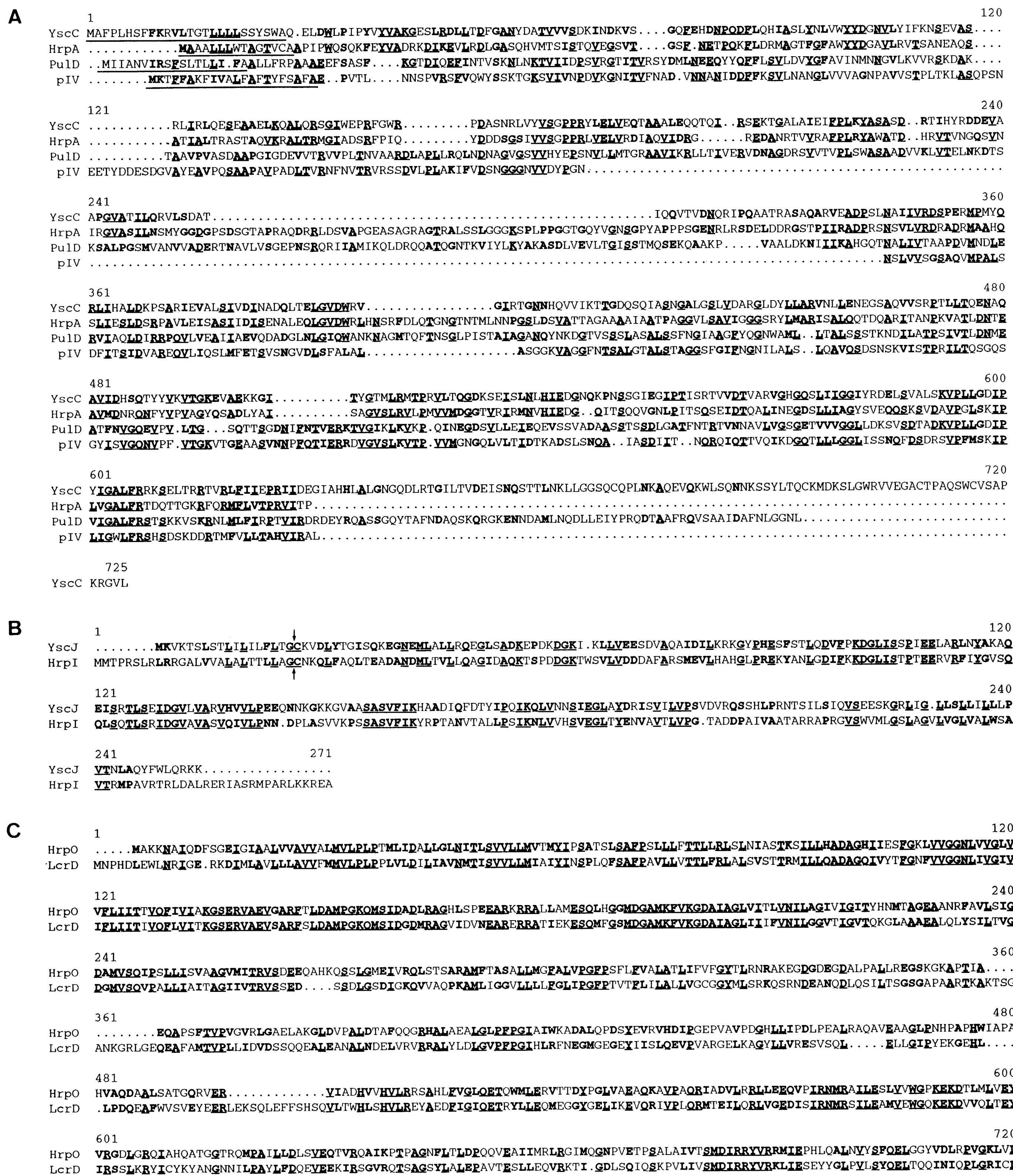

Fig. 2. Alignments of predicted *Pseudomonas solanacearum* Hrp proteins with *Yersinia* virulence proteins. Amino acids that are shared identically are in bold and underlined, and those shared conservatively are in bold. **A,** Sequence alignment between HrpA of *P. solanacearum*, YscC of *Yersinia enterocolitica* W22703 (Michiels *et al.* 1991), PulD of *Klebsiella pneumoniae* (d'Enfert *et al.* 1989), and the gene IV protein, pIV, of filamentous bacteriophage I2-2 (EMBL accession no. X14336). Potential N-terminal signal sequences are underlined. **B,** Sequence alignment between HrpI of *P. solanacearum* and YscJ of *Y. enterocolitica* W22703 (Michiels *et al.* 1991). Probable lipoprotein-specific cleavage sites are indicated by arrows. **C,** Sequence alignment between HrpO of *P. solanacearum* and LcrD of *Y. pestis* KIM (Plano *et al.* 1991). The amino acid sequences of HrpA, HrpI, and HrpO have been submitted to GenBank.

(*Yersinia* outer membrane proteins) from the cytoplasm to the external medium, indicating an involvement in the export of Yops or in the specific control of this function (Michiels *et al.* 1991). It has been demonstrated that the product of *yscJ* is required for Yop secretion (Michiels *et al.* 1991), and LcrD is an inner membrane-bound regulator of the export process (Plano *et al.* 1991). YscC is predicted to be an outer membrane protein with a role in export because of its sequence homology with PulD of *K. pneumoniae* and pIV of filamentous bacteriophage I2-2 and by analogy to the roles of these proteins in the export of pullulanase and phage particles, respectively (Michiels *et al.* 1991; Pugsley *et al.* 1990; Russel 1991). The homology between proteins involved in Yop export and proteins encoded by the pullulanase operon is, however, limited to that between YscC and PulD. In fact, although several gram-negative bacteria have protein secretion pathways in which many of the components show sequence homology with components required for pullulanase secretion (Pugsley 1992), Yops are probably exported from *Yersinia* by quite a distinct mechanism. In particular, Yops do not contain a classical N-terminal signal sequence that is involved in the translocation of pullulanase across the inner membrane (Michiels and Cornelis 1991). Unlike other bacterial proteins in which the export signal is contained in the 50 C-terminal amino acids (Koronakis *et al.* 1989), the appropriate Yop export signal is in the N-terminal region of the protein (Michiels and Cornelis 1991).

With the high levels of sequence identity presented here, it is tempting to speculate that *hrp*-encoded proteins of *P. solanacearum* are involved in the export of one or more macromolecules. Because four additional ORFs are also predicted to code for membrane-associated proteins (unpublished data), we envisage that these *hrp* genes are responsible for the production of a large export complex. Among the 18 *hrp* ORFs, the homology between HrpA and PulD was the only homology found to the pullulanase operon, and no homologies to proteins involved in other secretion pathways were detected. This, and the fact that HrpA and YscC are more closely related to each other than either is to PulD, implies that the hypothesized *hrp* export system may be functionally most similar to the Yop export system.

Interspecies conservation of *hrp* genes. Southern blot analysis showed that *hrpA* and *hrpI* probes hybridized to the *hrp* gene cluster of *X. c.* pv. *campestris* (Arlat *et al.* 1991) (Fig. 3A,B) and that an *hrpO* probe hybridized to the *hrp* gene clusters of *X. c.* pv. *campestris* (Arlat *et al.* 1991), *E. amylovora* (Barny *et al.* 1990), and *P. s.* pv. *phaseolicola* (Rahme *et al.* 1991) (Fig. 3C). This is the first report of *hrp* gene homology between *P. solanacearum* and bacteria from the species *P. syringae* and the genus *Erwinia*. Given that *X. c.* pv. *campestris*, *E. amylovora*, and *P. s.* pv. *phaseolicola* are representatives of three groups of phytopathogenic bacteria, within each of which *hrp* gene functional conservation or homology has already been shown (Bonas *et al.* 1991; Laby and Beer 1990; Lindgren *et al.* 1988; Arlat *et al.* 1991), it may be a general feature of *hrp* gene clusters that they code for such an export system. In addition, two *hrp* genes of *P. s.* pv. *syringae* encode membrane-associated or secreted proteins (Huang *et al.* 1991).

The same three virulence determinants of *Yersinia* featured in this paper are shown to have sequence similarity with predicted Hrp proteins of *X. c.* pv. *vesicatoria* in an accompanying paper (Fenselau *et al.* 1992). We therefore compared the amino acid sequences of HrpA, HrpI, and HrpO with their *X. c.* pv. *vesicatoria* counterparts and found high levels of sequence similarity (data not shown). From the hybridization data, we were able to show that the *X. c.* pv. *vesicatoria* genes corresponding to *hrpA*, *hrpI*, and *hrpO* are located in equivalent positions in the *X. c.* pv. *vesicatoria hrp* gene cluster. This is completely consistent with the discovery by Arlat *et al.* (1991) that the *X. c.* pv. *campestris hrp* gene cluster is homologous and colinear over a continuous 19-kb region with the *P. solanacearum hrp* gene cluster and with the fact that *hrp* genes are functionally conserved among *X. campestris* pathovars (Bonas *et al.* 1991; Arlat *et al.* 1991). The good sequence similarities and the similar sizes and predicted characteristics of the corresponding *P. solanacearum* and *X. c.* pv. *vesicatoria* proteins (data not shown) indicate that we have correctly predicted the ORFs for HrpA, HrpI, and HrpO.

Role of protein export in phytopathogenesis. As extracellular molecules, Yops mediate the interaction between

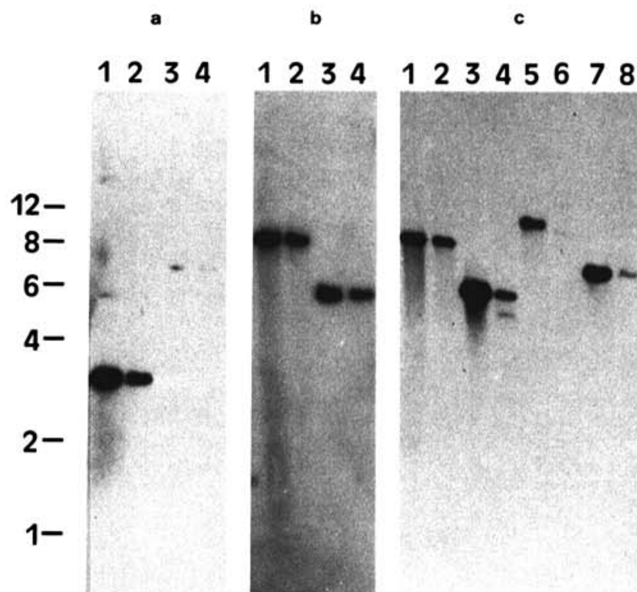

Fig. 3. Southern blot hybridizations of restricted plasmid and genomic DNA of different phytopathogenic bacterial strains probed with internal fragments of **A,** *hrpA* (the 475 C-terminal base pairs [bp]); **B,** *hrpI* (a central region of 228 bp); **C,** *hrpO* (the N-terminal 540 bp). Lanes 1,3,5,7: plasmid DNA containing *hrp* genes; and lanes 2,4,6,8: total genomic DNA. Plasmid and genomic DNA from the same bacterium are restricted with the same enzyme. Lanes 1,2: *Pseudomonas solanacearum* DNA (Boucher *et al.* 1987; Arlat *et al.* 1992) digested with *Eco*RI; 1A,B, pVir2; 1C, pAFE8; 2, GMI1000. Lanes 3,4: *Xanthomonas campestris* pv. *campestris* DNA (Arlat *et al.* 1991); A, digested with *Eco*RI, and B and C, digested with *Bam*HI; 3, pIJ3225; 4, 8004. Lanes 5,6: *Erwinia amylovora* DNA (Barny *et al.* 1990) digested with *Bam*HI; 5, pPV153; 6, CFBP1430. Lanes 7,8: *P. s.* pv. *phaseolicola* DNA (Rahme *et al.* 1991) digested with *Sst*I; 7, pPL6; 8, NPS3121. Approximately equal amounts of DNA were loaded (2 μg), except for the *P. solanacearum* plasmids pVir2 and pAFE8, for which there is approximately 100 times less. Hybridization signals obtained with plasmid DNA correspond to *hrp* genes only and not to vector DNA.

Yersinia and animal cells, probably partly by disrupting the normal signal transduction machinery of the host cell (Bliska *et al.* 1991). By analogy, the *hrp* export system could secrete elicitors that interact directly or indirectly with plant cells to induce two possible outcomes, disease or the HR. Such elicitors would be responsible for mediating the changes in plant gene expression that are induced during the interactions between *P. solanacearum* and tobacco and that are dependent on the *hrp* gene (Godiard *et al.* 1991). The dual role of *hrp* genes and the differentiation of the two plant responses may reside in this export function, and, in fact, we can consider potential candidates that would be secreted elicitors of the HR. According to the elicitor-receptor model that describes the incompatible interaction between a pathogen and a plant (Keen 1991), specific elicitors are primary or secondary products of avirulence alleles in the pathogen and are recognized by receptors encoded by the complementary plant disease resistance genes. The products of bacterial avirulence genes are mostly predicted to be hydrophilic proteins (Keen and Staskawicz 1988), which are not good candidates for being membrane-bound elicitors. Interestingly, the phenotypic expression of race-specific avirulence is usually dependent on the *hrp* gene (Lindgren *et al.* 1988; Keen *et al.* 1990). By comparing the role of the *Yersinia* export system in the secretion of proteins by a mechanism that does not involve classical N-terminal signal sequences (Michiels and Cornelis 1991) and given that none of the characterized avirulence gene products are predicted to contain an N-terminal signal sequence (Keen and Staskawicz 1988), we propose that *hrp* gene products could be involved in the HR in the secretion of elicitors that are the primary products of avirulence alleles.

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