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# Characterization of the *hrp* Cluster from *Pseudomonas syringae* pv. *syringae* 61 and Tn*phoA* Tagging of Genes Encoding Exported or Membrane-Spanning Hrp Proteins

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**The cosmid pHIR11, obtained from *Pseudomonas syringae* pv. *syringae* 61, enables *P. fluorescens* to elicit the hypersensitive response (HR) in tobacco leaves. To locate functional loci, pHIR11 was mutagenized with Tn*phoA* in *Escherichia coli*, and transposon insertion derivatives were mobilized into *P. fluorescens* and screened for their HR phenotype. Forty-one unique insertions that affected HR elicitation were identified. HR$^-$ pHIR11::Tn*phoA* derivatives were transferred into the *P. s.* pv. *syringae* genome by marker exchange. Thirteen complementation groups were then defined by analysis of merodiploids containing all combinations of Tn*phoA* insertions in the chromosome and in pHIR11. *P. s.* pv. *syringae* mutants in 12 of the complementation groups (II-XIII) exhibited Hrp$^-$ phenotypes, including loss of the ability to cause disease or to multiply in bean, to elicit the HR in tobacco leaves, or to cause HR-associated ionic responses in suspension-cultured tobacco cells. *P. s.* pv. *syringae* mutations in complementation group I were phenotypically distinct from those in the *hrp* region, producing a delayed HR (necrosis appeared after 24-48 hr) in tobacco leaves and retaining virulence in bean leaves. Two genes, in complementation groups IV and X, respectively, were determined to encode exported or membrane-spanning proteins by the PhoA$^+$ (alkaline phosphatase) phenotype of Tn*phoA* insertions. Analyses of Hrp-PhoA hybrid proteins immunostained with anti-PhoA antibodies and assays of PhoA activity in mutant cultures indicated that the expression of both genes was derepressed in a minimal medium known to derepress other *hrp* genes. Various pairs of HR$^-$ *P. s.* pv. *syringae* mutants co-inoculated into tobacco leaves failed to elicit the HR despite the use of high levels of inoculum. The core region of the *hrp* cluster (complementation groups III-XII) was homologous with the *hrp* cluster from *P. s.* pv. *phaseolicola* NPS3121 but not with *hrpM* from *P. s.* pv. *syringae* PS9020, as determined by Southern blot hybridizations. These results suggest that 12 of the 13 complementation groups analyzed are involved in the production of a single factor or activity that is necessary for bacterial multiplication *in planta* and for elicitation of both the HR and disease symptoms. Production or deployment of this factor requires two Hrp proteins that are either exported or have periplasmic domains.**

*Additional keywords:* bacterial plant pathogenesis, $K^+/H^+$ exchange response.

The species *Pseudomonas syringae* van Hall is composed of related bacteria that have been classified into pathovars primarily according to their host range (Dye *et al.* 1980; Young *et al.* 1978). A given plant species inoculated with members of this group generally expresses one of two types of plant reactions. Disease reactions typically are indicated by water-soaked lesions on susceptible host species and are associated with the multiplication and spread of the bacterium within infected plants (Ercolani and Crosse 1966; Omer and Wood 1969; Young *et al.* 1974). Resistant reactions on nonhost plants as well as on resistant cultivars of a susceptible host, on the other hand, are typified by the hypersensitive response (HR). The HR is characterized by the rapid, localized necrosis of tissues in infected sites, accompanied by cessation of bacterial growth (Klement 1982).

There is evidence suggesting that the mechanisms that control disease susceptibility and the HR are related. Klement (1982) has noted that the development of the two types of plant reactions are similar except for their timing. Induction of an apparent plasmalemma $K^+/H^+$ exchange response (XR) by *P. s.* pv. *syringae* van Hall was associated with both incompatible and compatible bacterium-plant interactions (Atkinson *et al.* 1985b; Atkinson and Baker 1987). The XR of tobacco cells that proceeds rapidly after inoculation with incompatible pseudomonads (Atkinson *et al.* 1985a,b) has been shown to be correlated genetically with the HR. Tn*5*-induced mutants of *P. s.* pv. *syringae* that are unable to elicit the XR in tobacco cells are also unable to elicit the HR in tobacco leaves (Baker *et al.* 1987). Finally, large clusters of *hrp* (hypersensitive response and pathogenicity) genes (Lindgren *et al.* 1986) have been cloned from several bacteria (Bonas *et al.* 1991; Boucher *et al.* 1987; Huang *et al.* 1988; Beer *et al.* 1991; Panopoulos *et al.* 1985; Niepold *et al.* 1985). These genes control the ability of pathogens to cause diseases on their hosts and to elicit HR in nonhost plants. The *hrp* genes from *P. s.* pv. *phaseolicola* (Berkholder) Young *et al.* NPS3121 appear to be interchangeable and homologous among pathovars of *P. syringae*, based on analysis of marker-exchanged mutants and Southern hybridizations (Lindgren *et al.* 1988). This indicates that these genes are functionally equivalent and conserved. Rahme *et al.* (1991) published a detailed description of the genetic organization of the *P. s.* pv. *phaseolicola* NPS3121 *hrp* cluster, and Willis *et al.* (1991) reviewed knowledge of the *hrp* genes in all phytopathogenic bacteria.

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Some *hrp* genes have been sequenced. The predicted gene product of *hrp*S from *P. s.* pv. *phaseolicola* NPS3121 (Grimm and Panopoulos 1989) belongs to the family of two-component regulatory proteins, many of which control virulence in bacteria (Miller *et al.* 1989), and appears to regulate the expression of other *hrp* genes (Grimm and Panopoulos 1989; Lindgren *et al.* 1989). The predicted products of *hrp*M and a linked open reading frame from *P. s.* pv. *syringae* PS9020 showed no similarity to any known proteins (Mukhopadhyay *et al.* 1988). The biochemical basis of *hrp* cluster involvement in elicitation of plant reactions (HR, XR, and disease symptoms) by *P. syringae* remains elusive, and identification of the elicitor through gene manipulation has not been achieved.

We have reported that a cosmid clone, pHIR11, containing a 30-kb DNA fragment from *P. s.* pv. *syringae* 61 has a functional set of *hrp* genes enabling elicitation of the HR and XR in tobacco by *P. fluorescens* Migula (Huang *et al.* 1988). To further explore the genetic basis for the elicitation of multiple plant reactions, we have used Tn*phoA* mutagenesis (Manoil and Beckwith 1985) to define complementation groups in the *hrp* cluster, to determine whether any complementation groups are responsible for a subset of the several plant-reaction phenotypes conferred by the whole cluster, and to search for genes that encode exported or membrane-spanning proteins. We provide here the first evidence that two Hrp proteins are either exported beyond the cytoplasm or have periplasmic domains.

## MATERIALS AND METHODS

**Bacteria, plasmids, and growth conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. pHIR11 and pHIR11BB8 were propagated in *Escherichia coli* CC118. pRK2013 was maintained in *E. coli* HB101. *Pseudomonas* strains were grown on King's medium B (KB) broth or agar (King *et al.* 1954), or Hrp minimal medium (50 mM potassium phosphate buffer, 7.6 mM $(NH_4)_2SO_4$, 1.7 mM $MgCl_2$, 1.7 mM NaCl, 10 mM mannitol, and 10 mM fructose, pH 5.7) (Huynh *et al.* 1989) at 28° C. The alkaline phosphatase substrate used here was 40 μg/ml of 5-bromo-4-chloro-3-indolyl phosphate (XP). *E. coli* was cultured on LM medium (Hanahan 1983). Media were supplemented with appropriate antibiotics at the following concentrations: kanamycin, 50 μg/ml; nalidixic acid, 20 μg/ml; and tetracycline, 20 μg/ml.

**Recombinant DNA manipulations.** Restriction enzyme digestions, agarose gel electrophoresis, plasmid extractions, nick translations, and bacterial transformations ($CaCl_2$ procedure) were performed as described by Maniatis *et al.* (1982). Southern blot analysis was done with GeneScreen Plus (Du Pont, Wilmington, DE) in 50% formamide according to the manufacturer's instructions. Probes were labeled with [$^{32}$P] (>$10^6$ disintegrations per minute) by nick translation (Bethesda Research Laboratories, Gaithersburg, MD) before hybridization. Blots were washed with 2× SSC (300 mM NaCl, 30 mM sodium citrate), 1% sodium dodecyl sulfate (SDS) at 65° C unless indicated otherwise before exposing the blots to Kodak XAR-5 film at −70° C.

**Tn*phoA* mutagenesis of pHIR11 and pHIR11BB8.** pHIR11 and pHIR11BB8, harbored in *E. coli* CC118, were mutagenized with λ::Tn*phoA* (Manoil and Beckwith 1985) as described previously (Huang *et al.* 1988). Mutated plasmids were mobilized by triparental matings, either *en masse* or individually, into pseudomonads for plant reaction assays or detection of alkaline phosphatase activity.

**Bacterial mating and marker-exchange mutagenesis of strain 61.** Triparental matings were carried out by mixing recipient nalidixic acid-resistant (Nal$^r$) pseudomonads with donor *E. coli* CC118(pHIR11::Tn*phoA*) and helper strain *E. coli* HB101(pRK2013) at a ratio of 10:1:1 (Ditta *et al.* 1980). The mating mixture was spotted on KB agar and incubated at 28° C overnight. The mixture of cells was either directly streaked onto the selective medium (KB supplemented with nalidixic acid, kanamycin, and tetracycline), or suspended in 1 ml of water and then spotted on the KB selective medium in 10-μl droplets. For detection of alkaline phosphatase activity, mating mixtures containing *P. s.* pv. *syringae* transconjugants were also spotted on Hrp minimal medium or KB medium containing appropriate antibiotics and XP. Single-colony transfers were used to purify the transconjugants. *P. s.* pv. *syringae* transconjugants containing mutated pHIR11 or pHIR11BB8 derivatives were then mutagenized by marker-exchange as described previously (Huang *et al.* 1988).

**Growth of plants and tobacco cell cultures.** Tobacco (*Nicotiana tabacum* L. 'Samsun' or 'Xanthi') used in HR assays and 10-day-old beans (*Phaseolus vulgaris* L. 'Bush Blue Lake 274') used in pathogenicity assays were grown under greenhouse conditions and then transferred to the laboratory and maintained at room temperature for HR assays or at 28° C with 16 hr of illumination per day for pathogenicity assays. Tobacco cell suspension cultures were derived either from *N. tabacum* cv. Hicks as described by Atkinson *et al.* (1985a) or from *N. tabacum* NT1 (Paszty and Lurquin 1987).

**Plant reaction assays.** Bacteria were grown on KB agar containing appropriate antibiotics overnight, suspended in 10 mM potassium phosphate buffer (pH 7.0), and adjusted to appropriate cell densities ($10^8$ cells per milliliter for HR assay or $10^5$ cells per milliliter for pathogenicity assay). Plant inoculations were done by pricking leaves with a dissecting needle and then pressing the blunt end of a tuberculin syringe against the leaf surface while supporting the leaf with a finger (Baker *et al.* 1987). The same technique was used for tobacco and bean plants. The plants were observed within 24 hr for development of the HR in tobacco or over a period of several days for development of disease symptoms in bean. Bean leaves were also sampled to measure bacterial populations. In brief, samples of inoculated tissue were taken by excision with a 6-mm cork borer, and the bacteria released by grinding the tissue in a microcentrifuge tube as described by Bertoni and Mills (1987). The population values calculated were based on the mean from three plate counts for each of three independent inoculations. The XR of tobacco cells was assayed by monitoring the increase in medium pH (Atkinson *et al.* 1985b). Assay mixtures containing 5 × $10^8$ bacteria per milliliter, 0.05 g of tobacco cells per

milliliter, 0.175 M mannitol, 0.5 mM $K_2SO_4$, 0.5 mM $CaCl_2$, and 0.5 mM MES (morpholineethanesulfonic acid) were adjusted to pH 6.0 ± 0.05 with 0.1 M NaOH. The assay mixture was incubated at 25° C with constant shaking (150 rpm), and the pH was monitored periodically. Assay results were obtained from duplicate cultures.

**Table 1.** Bacterial strains and plasmids used in this study

| Designation | Relevant characteristics | Reference or source |
|---|---|---|
| *Escherichia coli* | | |
| HB101 | F′ *hsdS20 recA13 thr leu thi pro* Sm$^r$ | Maniatis *et al.* 1982 |
| CC118 | *recA1 phoAv20 thi rps rpsE rpoB ΔlacX74* | C. Manoil 1985 |
| DH5α | *endA1 hsdR17 gyrA96 recA1 relA Δ(argF-lacZYA)U169 ϕ80dlacZΔM15* | Bethesda Research Laboratories |
| *Pseudomonas fluorescens* 55 | Nal$^r$ | M. Sasser |
| *P.s.* pv. *syringae* | | |
| 61 | WT isolated from wheat, Nal$^r$ | M. Sasser |
| 61-2070 to 2098 | A series of Tn*phoA* mutants of strain 61 constructed by marker exchange | This study |
| 61-2078 | Mutant in above series that has Tn*phoA* in group IV and is PhoA$^+$ | This study |
| 61-2089 | Mutant in above series that has Tn*phoA* in group X and is PhoA$^+$ | This study |
| Plasmids | | |
| pRK2013 | IncP Tra RK2$^+$ *ΔrepRK2 repE1*$^+$, Kan$^r$ | Ditta *et al.* 1980 |
| pHIR11 | 30-kb fragment of *P. s.* pv. *syringae* 61 containing *hrp* cluster cloned in pLAFR3 | Huang *et al.* 1988 |
| pHIR11BB8 | 8.4-kb *Bam*HI-*Bgl*II fragment from pHIR11 subcloned into pLAFR3 | Huang *et al.* 1988 |
| pCPP2070 to 2098 | A series of pHIR11 derivatives containing Tn*phoA* insertions (the insertion in pCPP2078 is in pHIR11BB8) | This study |
| pCPP2078 | Member of the above series containing a PhoA$^+$ Tn*phoA* insertion in group IV | This study |
| pCPP2089 | Member of the above series containing a PhoA$^+$ Tn*phoA* insertion in group X | This study |
| pPL11 | 18-kb *Bam*HI-*Eco*RI fragment of *P. s.* pv. *phaseolicola* NPS3121 cloned into pLAFR3 | Lindgren *et al.* 1989 |
| pOSU3158 | 8.5-kb *Hin*dIII-*Xbo*I fragment in pVK102 cloned from *P. s.* pv. *syringae* PS9020 containing the *hrpM* locus and a Tn*5* insertion | D. Mills |

**Western blotting of Hrp-PhoA hybrid proteins.** Bacteria were grown in 5 ml of Hrp minimal broth for two days or KB broth for one day at 30° C, harvested by centrifugation, washed with 10 mM Tris buffer (pH 8.0), resuspended in 2 ml of the same buffer, then sonicated. Samples (800 μl) were precipitated with 5% trichloroacetic acid, washed with acetone, dissolved in 20 μl of 10 mM Tris-HCl buffer, and boiled with an equal volume of 2× loading buffer (0.625 M Tris, pH 6.8, 2% SDS, 10% glycerol, 2% β-mercaptoethanol) for 1 min. A 20-μl sample was subjected to SDS-10% polyacrylamide gel electrophoresis (PAGE) in a 0.75 mm thick gel in a Hoefer Mighty Small apparatus (Hoefer Scientific, San Francisco, CA.) Prestained molecular size standards (Bio-Rad Laboratories, Richmond, CA) were used to calibrate protein mobilities on the blot. After separation, the protein bands were transferred to a GeneScreen membrane (Du Pont) in a high-field electroblotting apparatus (Hoefer) as described by manufacturer's instructions. The transblotted filter was probed with anti-PhoA antiserum. Immunodetection of the bands was performed with an alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma, St. Louis, MO) as described by the manufacturer's instruction.

## RESULTS

**Determination of *hrp* loci and complementation groups by Tn*phoA* mutagenesis.** One hundred and twenty-eight Tn*phoA*-containing pHIR11 derivatives were mobilized from *E. coli* CC118 to *P. fluorescens* 55 by triparental matings. Approximately 32% of the transconjugants were unable to elicit the HR on the tobacco leaves. Tn*phoA* insertions in all HR$^-$ and some HR$^+$ derivatives of pHIR11 were mapped by restriction enzyme digestions (Fig. 1). This revealed that a 25-kb DNA fragment was involved in the elicitation of the HR in *P. fluorescens*.

To define complementation groups, merodiploids of *P. s.* pv. *syringae* 61 were constructed. First, all HR$^-$ Tn*phoA* mutations in pHIR11 were transferred into the strain 61 chromosome by marker-exchange to generate a collection of mutants. Second, merodiploids were constructed by mobilizing various Tn*phoA*-mutated derivatives of pHIR11 into the mutants. Based on the HR phenotypes on tobacco leaves caused by these merodiploids, 13 complementation groups were defined (Fig. 1).

**Phenotypes of mutations in each complementation group in the *hrp* cluster.** Any insertion of Tn*phoA* in complementation groups II–XIII inactivated all *hrp*-associated phenotypes in *P. s.* pv. *syringae* 61, including the HR on tobacco leaves, XR in tobacco cell suspensions, and disease and multiplication on bean leaves (Fig. 1). In contrast, mutations in group I, for instance mutant 61-2070, slightly affected plant responses. The HR was delayed and the XR slightly reduced, but bacteria still produced symptoms and grew on bean leaves (Figs. 2 and 3). The growth curves for *P. s.* pv. *syringae* strains 61 and 61-2070 in bean leaves were similar (Fig. 2), but elicitation of the XR by 61-2070 was reduced 65% relative to the wild type at 6 hr after

inoculation .(Fig. 3). In contrast, mutation 2089 in complementation group X abolished the ability of the bacteria to multiply in bean leaves (Fig. 2) or to elicit the XR in tobacco cell suspensions (Fig. 3). Other HR$^-$ mutants of *P. s.* pv. *syringae* 61 showed reductions in the ability to multiply and elicit the XR similar to mutant 61-2089 (data not shown).

**Co-inoculation of *P. s.* pv. *syringae* derivatives containing complementing Hrp$^-$ mutations.** To test the possibility that extracellular *hrp* products would permit restoration of the HR phenotype by co-inoculation, pairs of HR$^-$ *P. s.* pv. *syringae* mutants representing all possible combinations of complementation groups were infiltrated into tobacco leaves. Despite the use of high levels of inoculum ($10^8$ cells per milliliter), in no case was the HR restored.

**Homology of pHIR11 to the *hrp* genes of *P. s.* pv. *phaseolicola* NPS3121 and *P. s.* pv. *syringae* PS9020.** To determine whether pHIR11 exhibits homology to the *hrp* genes from two other pseudomonads, restriction digests of pHIR11 were probed with [$^{32}$P]-labeled plasmids containing the cloned *hrp* cluster from *P. s.* pv. *phaseolicola* NPS3121, pPL11 (Lindgren *et al.* 1989), or the *hrpM* locus from *P. s.* pv. *syringae* PS9020, pOSU3158 (Mukhopadhyay *et al.*). Strong hybridization under high stringency conditions was observed between pHIR11 and pPL11 (Fig. 4, panel A), but no hybridization was seen under any condition when pOSU3158 was the probe (Fig. 4, panel B). pPL11 hybridized with the 2.6-kb *Eco*RI-*Bgl*II, 4.3-kb *Bgl*II-*Bgl*II, 6.0-kb *Bgl*II-*Eco*RI, 1.6-kb *Eco*RI-*Eco*RI, and 3.2-kb *Eco*RI-*Bgl*II fragments internal to pHIR11 (lanes 2 and 3). No hybridization was observed with the 3.7-kb *Bam*HI-*Eco*RI, 2.3-kb *Eco*RI-*Eco*RI and 6.3-kb *Bgl*II-*Eco*RI fragments of pHIR11. These results indicate that pHIR11 contains the core *hrp* cluster (from regions III–XII) that is found in *P. s.* pv. *phaseolicola* NPS3121.

**Screening for Tn*phoA* insertions in *hrp* genes that encode exported proteins.** Three hundred Tn*phoA*-mutated derivatives of pHIR11 and pHIR11BB8 were mobilized into *P. s.* pv. *syringae* 61 and then screened for alkaline phosphatase activity indicative of insertions in an exported protein. Two mutated cosmids, pCPP2089 and pCPP2078, possessed alkaline phosphatase activity and produced blue colonies on plates of Hrp minimal medium containing XP, but not in KB containing XP (Fig. 5). The Tn*phoA* insertions were restriction mapped. pCPP2089 contains the

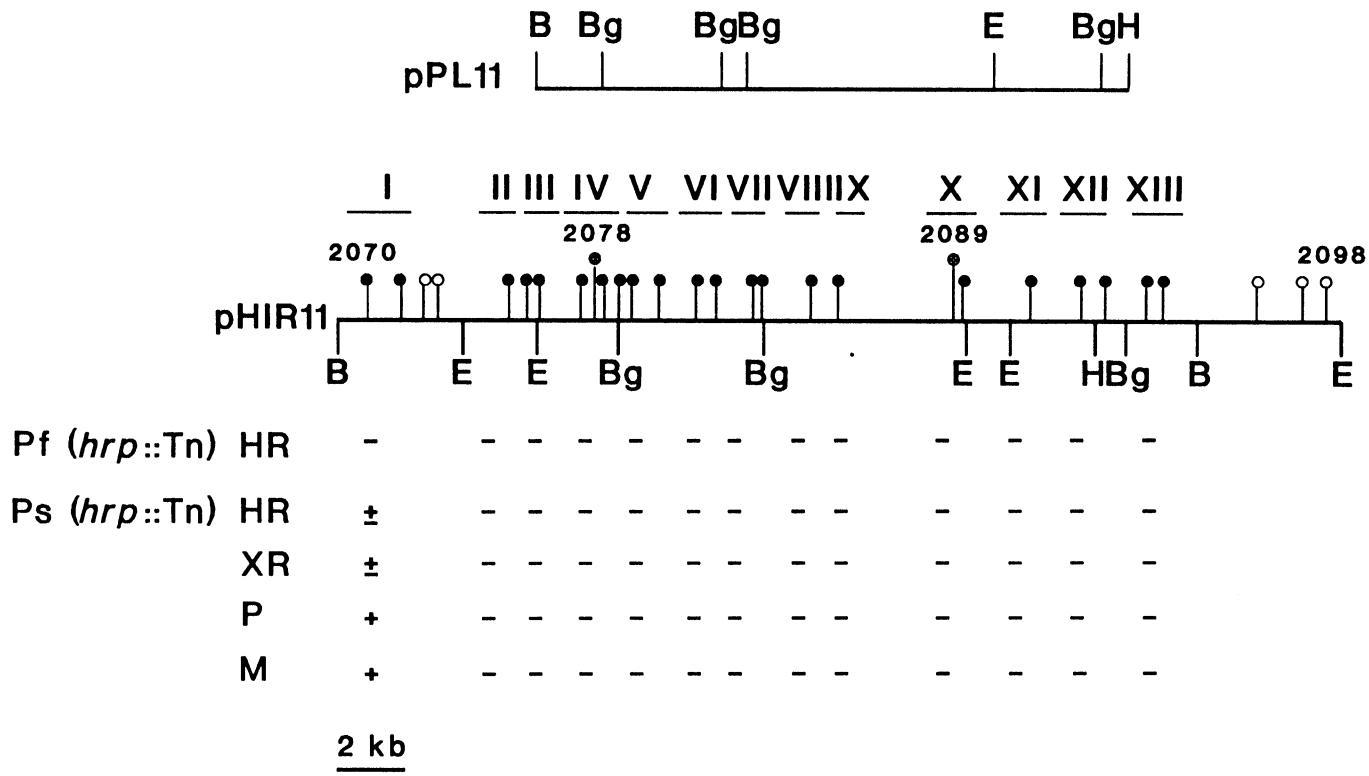


**Fig. 1.** Restriction map of Tn*phoA* insertions and complementation groups in pHIR11 and plant reaction phenotypes resulting from mutations in each complementation group. Open circles indicate that Tn*phoA* insertions had no affect on the hypersensitive response (HR) elicited by *Pseudomonas fluorescens* carrying the mutated derivative. Solid circles indicate that Tn*phoA* insertions resulted in loss of HR activity in *P. fluorescens*. Hatched circles denote insertions with alkaline phosphatase activity (the 2078 insertion is in subclone pHIR11BB8). pHIR11::Tn*phoA* derivatives are numbered in a consecutive series that begins with pCPP2070 and runs from left to right on the map up to pCPP2098. These mutated derivatives were marker-exchanged into *Pseudomonas syringae* pv. *syringae* 61, and mutants were assigned the number corresponding to the plasmid. Complementation groups were determined by merodiploid analysis of all possible combinations of mutations in the *P. s.* pv. *syringae* 61 chromosome and in pHIR11::Tn*phoA* derivatives. The restriction map of the *hrp* cluster in pPL11 from *P. s.* pv. *phaseolicola* NPS3121 (Lindgren *et al.* 1989) is shown above the map of pHIR11 (relative orientation is not known). Symbols: E, *Eco*RI; Bg, *Bgl*11; H, *Hin*dIII; B, *Bam*HI; Roman numerals represent different complementation groups; HR, hypersensitive response in tobacco; XR, plasmalemma $K^+/H^+$ exchange response in tobacco cell suspensions; P, disease symptoms in bean; M, multiplication in bean; +, normal reaction; ±, reduced or delayed reaction; −, no reaction.

insertion in complementation group X in pHIR11. pCPP2078 contains the insertion in group IV and is carried in pHIR11BB8. These Tn*phoA*-induced mutations were marker-exchanged into the *P. s.* pv. *syringae* 61 chromosome. The resulting mutants, 61-2089 and 61-2078, also produced alkaline phosphatase activity in the Hrp minimal medium only (Fig. 5).

**Western blotting of Hrp-PhoA proteins immunostained with anti-PhoA antibody.** To confirm that the Hrp-PhoA proteins were produced only in minimal medium and to determine the molecular sizes of the hybrid proteins, proteins in lysed cultures of *P. s.* pv. *syringae* 61 carrying pCPP2089 and pCPP2078 were resolved by SDS-PAGE and immunostained with anti-PhoA antibodies. Hybrid proteins were detected when bacteria grew in Hrp minimal medium (Fig. 6). Hybrid proteins could not be detected with anti-PhoA antibodies in bacteria grown in KB medium (data not shown). The molecular sizes of the hybrid proteins were 60 and 65 kD respectively. Mature PhoA (~47 kD) was also observed, presumably as a degradation product of Hrp-PhoA hybrids.

## DISCUSSION

The HR has been experimentally alluring for decades because of its rapidity and its association with disease resistance in many different plant-pathogen systems. However, the molecular basis for this response of higher plants to inoculation with an incompatible pathogen remains elusive. Genetic analysis of the ability of *P. syringae* pathovars to elicit the HR has revealed two important features. First, it appears that the same genes required for HR elicitation are also required for pathogenicity (Niepold *et al.* 1985; Lindgren *et al.* 1986). Second, the HR and pathogenic phenotypes of *P. syringae* require the action of several of these *hrp* genes (Cuppels 1986; Huang *et al.* 1988; Lindgren *et al.* 1986). We had previously isolated from *P. s.* pv. *syringae* 61 a gene cluster with the unusual ability to confer HR-eliciting activity to the nonpathogenic bacterium, *P. fluorescens* 55 (Huang *et al.* 1988). We now have found that 1) the core of this *hrp* cluster is homologous with a cluster of *hrp* genes from *P. s.* pv. *phaseolicola* NPS3121, which was previously shown to be conserved in other *P. syringae* pathovars (Lindgren *et al.* 1988); 2) that linked to this cluster of conserved *hrp* genes is a second locus (complemention group I), which is required for the HR phenotype in *P. fluorescens* but not in *P. s.* pv. *syringae* 61; 3) that complementation groups II–XIII, which we have defined in the cloned *P. s.* pv. *syringae* 61 *hrp* cluster, are necessary for a variety of plant-associated phenotypes in pseudomonads; and 4) that two *hrp* genes, in complementation groups IV and X, encode exported or membrane-spanning proteins, and the expression of these genes is derepressed in Hrp minimal medium.

Based on Southern blot hybridizations of pHIR11 with the *hrp* cluster carried on pPL11 from *P. s.* pv. *phaseolicola* NPS3121 (Lindgren *et al.* 1989), we have found that pHIR11 contains an approximately 18-kb region that is broadly conserved in the *P. syringae* pathovars. Similarly,

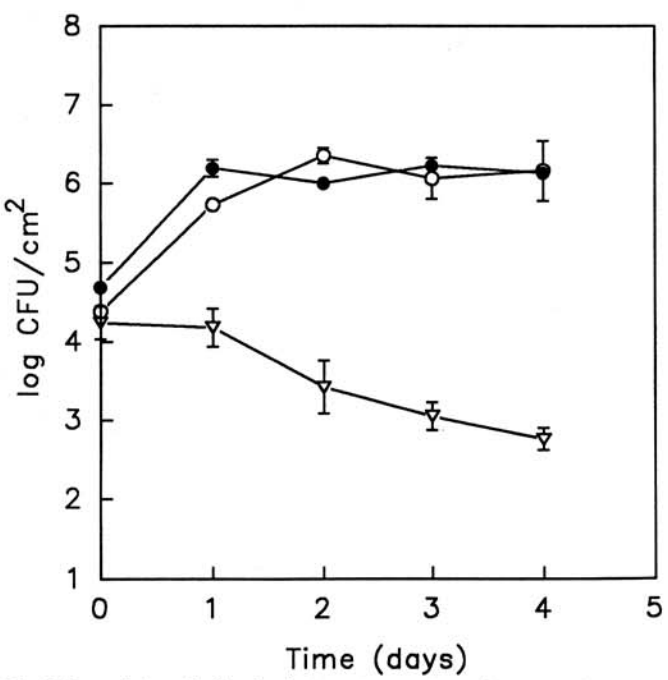


**Fig. 2.** Bacterial multiplication in bean leaves. Bacterial populations were monitored by dilution plating of crushed tissue at the indicated times after inoculation. Values represent the mean and standard deviation from three plate counts for each of three independent inoculations. Symbols: solid circles, wild type *Pseudomonas syringae* pv. *syringae* 61; open circles, complementation group I mutant *P. s.* pv. *syringae* 61-2070; open triangles, group X mutant *P. s.* pv. *syringae* 61-2089.

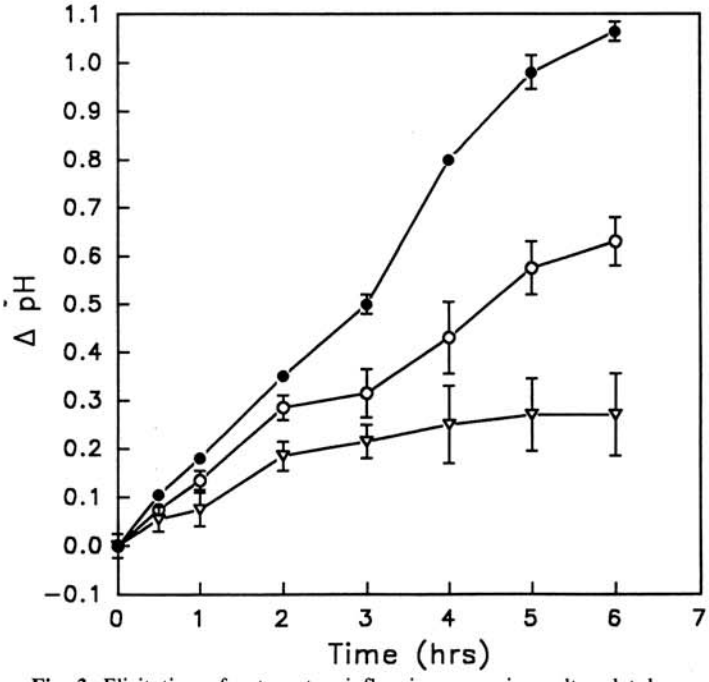


**Fig. 3.** Elicitation of net proton influx in suspension-cultured tobacco cells inoculated with *Pseudomonas syringae* pv. *syringae* 61 and mutants carrying Tn*phoA* in complementation groups I and X. The pH of the cell culture medium was monitored after inoculation with bacteria at $10^8$ cells per milliliter. Values represent the mean and standard deviation from duplicate samples. Symbols: solid circles, wild type *P. s.* pv. *syringae* 61; open circles, complementation group I mutant *P. s.* pv. *syringae* 61-2070; open triangles, group X mutant *P. s.* pv. *syringae* 61-2089.

a 14-kb *Eco*RI fragment in pHIR11 also hybridizes at low stringency with a *hrp* cluster from *Erwinia amylovora*, which confers the HR phenotype to *E. coli* (Beer *et al.* 1991). These DNA hybridization studies suggest that plant pathogenic bacteria have a highly conserved *hrp* region. The lack of homology between pHIR11 and *hrpM* of *P. s.* pv. *syringae* PS9020 suggests that this locus, if present in *P. s.* pv. *syringae* 61, is not linked to the *hrp* cluster carried on pHIR11. The *hrpM* locus in *P. s.* pv. *phaseolicola* NPS3121 also is not linked to the *hrp* cluster carried on pPL11 (Fellay *et al.* 1991). It thus appears likely that the *hrp* clusters of other *P. syringae* strains could confer the HR phenotype to *P. fluorescens* if they included all of the complementation groups defined for pHIR11 (II and XIII are missing from pPL11) and a functional equivalent of the group I locus.

The nature of the group I locus, which we are designating *hrm* (hypersensitive response modulator), remains unclear. One possibility is that *hrm* may have an *avr*-like function that interacts with the product of a corresponding resistance gene in tobacco. This observation is consistent with our previous observation that pHIR11BB6, the subclone from pHIR11 containing the *hrm* locus, converted *P. s.* pv. *tabaci* into an incompatible pathogen on its host, tobacco (Huang *et al.* 1988). However, this is difficult to test without tobacco cultivars that give differential reactions to $Hrp^+$ pseudomonads carrying *hrm*. Another possibility is that *hrm* affects the expression of *hrp* such that plant reaction phenotypes are qualitatively altered in *P. fluorescens*, and only quantitatively altered in *P. s.* pv. *syringae* 61. This region is being sequenced to gain further insight into its nature (S. Heu and S. W. Hutcheson, in preparation).

Rahme *et al.* (1991) determined that the *hrp* cluster in *P. s.* pv. *phaseolicola* NPS3121 spans 22 kb and is composed of seven complementation groups, as determined by the ability of various *hrp* subclones to restore the HR elicitation phenotype to merodiploids carrying transposon insertions in chromosomal *hrp* genes. Our approach has been to define complementation groups by using merodiploids in which chromosomal *hrp* mutations were complemented with pHIR11 derivatives carrying Tn*phoA* insertions (de Bruijn and Lupski 1984). Interestingly, we have identified 13

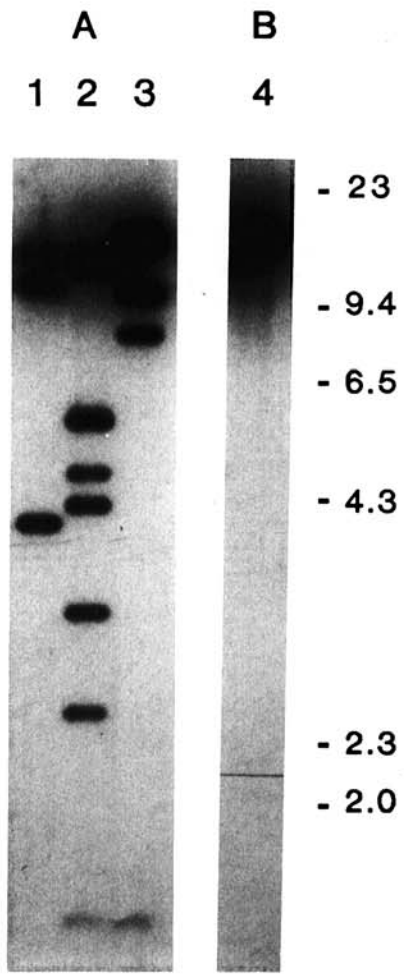


**Fig. 4.** DNA hybridization between the cloned *hrp* clusters in pHIR11 from *Pseudomonas syringae* pv. *syringae* 61 and in pPL11 from *P. s.* pv. *phaseolicola* NPS3121 or *hrp*M in pOSU3158 from *P. s.* pv. *syringae* PS9020. The isolated plasmids were digested with the indicated restriction enzymes and electrophoresed through a 0.7% agarose gel. DNA fragments were transferred to GeneScreen Plus and hybridized with heat-denatured, [$^{32}$P]-labeled pPL11 (panel A) or pOSU3158 (panel B). Lanes: 1, *Eco*RI-digested pPL11; 2, *Eco*RI- and *Bgl*II-digested pHIR11; 3 and 4, *Eco*RI-digested pHIR11. Sizes are shown in kilobases.

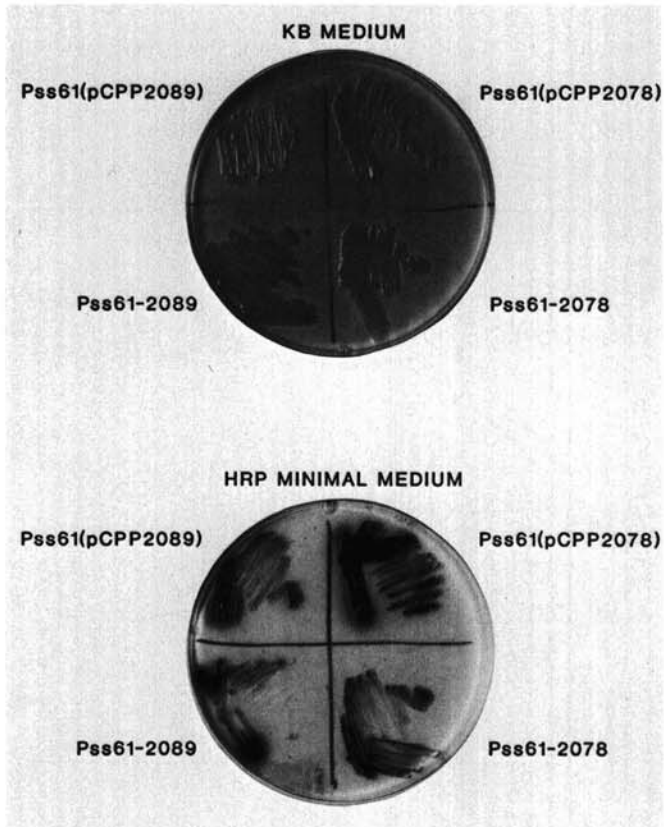


**Fig. 5.** Alkaline phosphatase activity in *Pseudomonas syringae* pv. *syringae* 61 derivatives carrying *hrp*::Tn*phoA* fusions and grown on Hrp-derepressing medium. Bacteria were streaked on the indicated agar media supplemented with 5-bromo-4-chloro-3-indolyl phosphate, a substrate from which alkaline phosphatase releases a blue chromogen. The plates were photographed through a Wratten 23A filter (Kodak, Rochester, NY). *P. s.* pv. *syringae* 61 cultures lacking these plasmids revealed no alkaline phosphatase activity on either medium (not shown).

complementation groups in pHIR11 by this approach. Unless the organization of the two *hrp* clusters is substantially different, our results suggest that Tn*phoA* insertions in *P. s.* pv. *syringae* 61 are not polar and that these complementation groups represent cistrons rather than operons. We are now sequencing several regions in pHIR11 to gain further insight into the organization and function of the *hrp* genes.

Our finding that mutations in any of the *hrp* complementation groups II-XIII abolishes the ability of pseudomonads to multiply or cause any reactions in plants suggests that a single product or activity derived from this region is responsible for all of these phenotypes. Again, somewhat different results were obtained with *P. s.* pv. *phaseolicola* NPS3121, where mutations in *hrpC* prevented elicitation of the HR in tobacco leaves but allowed bacteria to produce delayed and attenuated symptoms and to multiply to a level $10^2$- to $10^3$-fold lower than that of the wild type strain in bean leaves (Rahme *et al.* 1991). Our data indicate that a locus with the phenotype of *hrpC* is absent from pHIR11, although the 18-kb DNA segment containing *hrpC* in pPL11 is homologous to pHIR11.

The failure of *P. s.* pv. *syringae* 61 derivatives that contain complementing *hrp* mutations to elicit the HR when coinoculated in tobacco leaves argues against extracellular intermediates or a combination of extracellular factors being involved in HR elicitation. However, the expectation that the HR is elicited by an activity at the surface of the bacterium has lead us to use Tn*phoA* to survey the *hrp* cluster for genes that encode exported or membrane-spanning proteins. Tn*phoA* exploits the ability of the *E. coli phoA* product to be enzymatically active only when exported across the cytoplasmic membrane to the periplasmic space or the bacterial milieu (Manoil and Beckwith 1985). Tn*phoA* carries a *phoA* gene that lacks signal peptide sequences and accordingly can be used as a sensor for exported proteins carrying signal peptides. We found that two different complementation groups have yielded Hrp-PhoA hybrids with alkaline phosphatase activity. It should be noted, however, that any exported Hrp proteins that are secreted by a Sec-independent pathway and lack amino-terminal signal peptides would not be detected by this approach (Pugsley 1989).

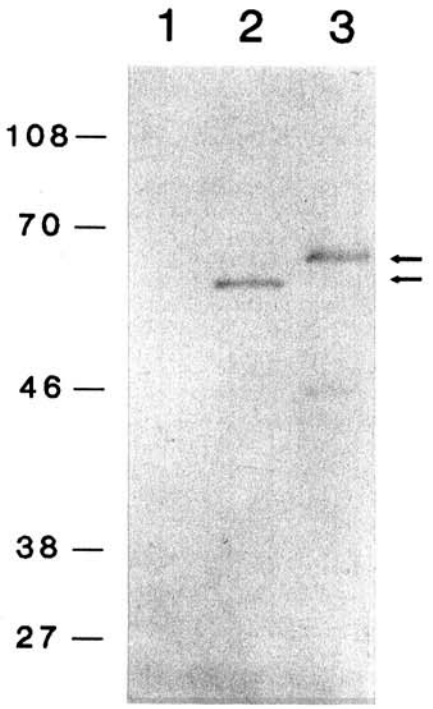


**Fig. 6.** Western blot of Hrp-PhoA hybrids produced by *Pseudomonas syringae* pv. *syringae* 61 cells carrying *hrp*::Tn*phoA* plasmids. Proteins in sonicated extracts of bacteria grown in Hrp minimal medium were electrophoresed in a 10% polyacrylamide gel, transferred to a nylon membrane, and then immunostained with anti-PhoA antibodies as described in the text. Lanes: 1, *P. s.* pv. *syringae* 61 (pHIR11); 2, *P. s.* pv. *syringae* 61 (pCPP2089); and 3, *P. s.* pv. *syringae* 61 (pCPP2078). Sizes are shown in kilodaltons.

PhoA can also be used as a sensitive reporter of target gene expression. We have used assays for alkaline phosphatase activity or PhoA antigen production to determine that the *hrp* genes in complementation groups IV and X are derepressed in the *hrp*/*avr* expression minimal medium defined by Huynh *et al.* (1989). Thus, the regulation of these two *P. s.* pv. *syringae* 61 *hrp* genes appears similar to that of the *hrp* genes of *P. s.* pv. *phaseolicola* NPS3121 (Fellay *et al.* 1991) and *E. amylovora* (Beer *et al.* 1991). This observation is also consistent with the report of Yucel *et al.* (1989) that *P. s.* pv. *syringae* 61 cells cultured in nitrogen-deficient media elicit an accelerated XR relative to cells cultured in rich media.

The protein products of complementation groups IV and X could have four potential roles in the production of the elusive elicitor of the HR: as membrane-spanning environmental sensors regulating *hrp* gene expression; as envelope proteins involved in elicitor secretion; as envelope proteins modifying or activating the elicitor; and/or as a protein that is secreted across the outer membrane and then functions as the elicitor itself, presumably after modification by cytoplasmic Hrp proteins. We are currently characterizing these two *hrp* genes and their products to differentiate among these possibilities and gain insight into the nature of the elicitor.

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