JOURNAL OF BACTERIOLOGY, Mar. 1992, p. 1734–1741
0021-9193/92/061734-08$02.00/0

Vol. 174, No. 6

Organization and Environmental Regulation of the *Pseudomonas syringae* pv. syringae 61 *hrp* Cluster

YINGXIAN XIAO, YANG LU,† SUNGGI HEU, AND STEVEN W. HUTCHESON*

Department of Botany and Center for Agricultural Biotechnology of the Maryland Biotechnology Institute, University of Maryland, College Park, Maryland 20742-5815

Received 16 July 1991/Accepted 15 January 1992

The ability of *Pseudomonas syringae* pv. syringae 61 to elicit the hypersensitive response in nonhost plant species has been linked to a cluster of *hrp/hrm* genes whose expression appears to be environmentally regulated. To understand the genetic organization of this *hrp/hrm* gene cluster and its expression during the interaction with nonhost plant species better, we constructed a set of chromosomal *hrp-uidA* fusions in *P. syringae* pv. syringae 61 by Tn*5-gusA1* mutagenesis of the cloned *hrp/hrm* gene cluster and transferred them into the genome by marker exchange mutagenesis. Complementation analysis employing plasmid-borne Tn*5-gusA1* insertions and previously characterized chromosomal Tn*phoA* mutations defined at least eight apparent transcriptional units within the *hrp/hrm* cluster, several of which were multicistronic. The expression of *hrp-uidA* fusions in seven of these apparent *hrp* transcriptional units increased following inoculation into tobacco leaves. Enhanced expression from a representative fusion was detected 1 h after inoculation of tobacco leaves. The induction observed in planta was similar to the levels detected following culture of the bacteria in minimal-salts medium: irrespective of the carbon source. Complex amino acid sources, such as peptone, repressed the expression of *P. syringae* pv. syringae 61 *hrp* genes at levels exceeding 0.028%. The results indicate that enhanced expression of *hrp* genes occurs early in the interaction with nonhost plant species in an apparent response to altered nutritional conditions.

The hypersensitive response (HR) is a rapid, localized necrosis of plant tissue that is observed when strains of *Pseudomonas syringae* are inoculated into nonhost plant species or resistant varieties of susceptible plant species (24). The HR appears to be a manifestation of localized plant defense mechanisms initiated by a postulated recognition event between *P. syringae* and a resistant host plant (7, 20–22). The recognition process requires a 2- to 4-h induction stage during which the plant response is sensitive to inhibitors of bacterial transcription and translation (24, 41). This suggests that bacterial gene expression induced in response to an undefined environmental signal during the initial 2 to 4 h of the interaction may be necessary to elicit the HR (17, 22, 41). Yucel et al. (53) used the timing and antibiotic sensitivity of an HR-associated response of cultured tobacco cells to show that some *P. syringae* strains may respond to nutritional conditions during the induction stage. Culturing of *P. syringae* pv. pisi or *P. syringae* pv. syringae 61 in minimal-salts medium for 3 h or more abolished the induction stage for the plant response. This effect could be reversed by addition of peptone to the pretreatment medium.

Huang et al. (16) reported the isolation of a large gene cluster from *P. syringae* pv. syringae 61, carried by cosmid pHIR11, that enables nonpathogenic bacteria, such as *Pseudomonas fluorescens* and *Escherichia coli*, to initiate the HR in several plant species (11, 15, 16, 17a). This observation suggests that pHIR11 carries all of the essential genes necessary for elicitation of the HR which are absent from the genome of the nonpathogenic bacteria. Previous genetic analyses have shown that this gene cluster consists of at least 12 *hrp* complementation groups that also function in bacterial pathogenicity during compatible interactions and the *hrmA* locus (previously designated complementation group I) that appears to modulate the phenotypic expression of the *hrp* genes during incompatible interactions (15). Elicitation of the HR by bacteria carrying this cluster appears to occur via the concerted action of the entire *hrp/hrm* cluster (11, 15, 17a). The *P. syringae* pv. syringae 61 *hrp/hrm* genes are thus likely genes to be induced during the induction stage of the HR. Enhanced expression of several *hrp* genes has been reported during pathogenesis of *P. syringae* pv. phaseolicola in its susceptible host, bean plants (9, 27, 38). Expression of *P. syringae hrp* genes during the interaction with nonhost plant species has not been established.

To understand the regulation of the *P. syringae* pv. syringae 61 *hrp* gene cluster and its role in eliciting the HR better, we (i) constructed a set of *hrp*::Tn*5-gusA1* derivatives; (ii) partially defined the transcriptional organization of the cluster; (iii) characterized the temporal expression of a representative *hrp* locus in a nonhost plant species, *Nicotiana tabacum* L. (tobacco); and (iv) investigated the influence of plant factors and culture conditions on the expression this *hrp* locus.

(Preliminary reports of these studies have appeared elsewhere [49, 50].)

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Plasmids were propagated in *E. coli* DH5α or CC118 prior to transfer into *P. syringae* pv. syringae 61. *E. coli* strains were grown in LB medium (10) at 37°C. *P. syringae* pv. syringae strains were routinely grown at 28°C in KB medium (23) until the culture reached mid-logarithmic growth (5×10^8 cells per ml). M63 minimal-salts medium (44) was used with 0.2% mannitol as a carbon source (M63M)

* Corresponding author.
† Present address: Department of Pharmacology, Georgetown University Medical Center, Washington, DC 20007.

TABLE 1. Bacterial strains, plasmids, and phage

Strain, plasmid, or phage	Relevant characteristic(s)	Reference or source
E. coli		
DH5α	*endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 recA1 gyrA96 relA1* Δ(*argF-lacZYA*)*U169* φ80d*lacZ*DM15	Bethesda Research Laboratories
CC118	*recA1 phoAv20 thi rps rpsE rpoB lacX74*	33
LE392	F⁻ *hsdR514 supE44 supF58 lacY1 galK2 galT22 metB1 trpR55* λ⁻	5
P. syringae pv. syringae		
61	Nx^r	16
61-20XX	Strain 61 derivative carrying mapped *hrp*::Tn*phoA* mutations	15
61-L14X	Strain 61 derivative carrying a *lux-nptI* insertion	This report
61-5XXX	Strain 61 derivative carrying *hrp*::Tn*5-gusA1* mutations	This report
Plasmids		
pLAFR3	Tc^r; cosmid derivative of pRK2	45
pHIR11	30-kb fragment of *P. syringae* pv. syringae 61 containing *hrp/hrm* cluster cloned into pLAFR3	16
pHIR11-5XXX	pHIR11 derivative carrying mapped Tn*5-gusA1* insertions	This report
Phage λ58::Tn*5-gusA1*	λ::Tn*5-gusA1* b221 Oam29 Pam80 Tc^r Kn^r	42

unless indicated otherwise. Nitrogen equivalents were calculated from the reported molar fraction of nitrogen in the compound used as the nitrogen source. Peptone was assumed to be 10% nitrogen. Media were supplemented with antibiotics, when indicated, at the following concentrations (micrograms per milliliter): kanamycin, 50; nalidixic acid, 50; tetracycline, 20.

General DNA manipulations. Plasmid DNA was isolated and manipulated by using standard techniques (40). Restriction enzymes, T4 DNA ligase, mung bean nuclease, and related reagents were purchased from Bethesda Research Laboratories, Gaithersburg, Md., and used in accordance with the manufacturer's recommendations. Calf intestine alkaline phosphatase was purchased from Boehringer Mannheim (Indianapolis, Ind.).

Plasmids were introduced into *E. coli* by transformation (40) or electroporation (8). For electroporation, bacteria were grown to the mid-logarithmic phase (optical density at 600 nm, 0.3 to 0.4). Cells were harvested and washed twice with cold 10% (wt/vol) glycerol. The washed cells were suspended in 10% glycerol to about 10^{10} bacteria per ml and stored at −70°C. A preparation of isolated plasmid DNA (1 to 10 ng/ml) was mixed with 50 μl of the gently thawed bacterial suspension and pulsed once at 10 kV/cm with a Gene Pulser (Bio-Rad, Richmond, Calif.) set at 25 μF of capacitance and equipped with a 200-Ω pulse controller. One milliliter of LB broth was immediately added, and the cells were incubated for 1 h prior to plating on KB agar containing the appropriate antibiotics. Plasmids were mobilized from *E. coli* strains into *P. syringae* pv. syringae 61 by triparental matings employing pRK2013 as the helper plasmid (6).

Physical mapping of pHIR11 was completed by standard procedures (37). Cleavage sites for the restriction enzymes were mapped initially in subclones of pHIR11 by single-, double-, and triple-digestion methods. These sites were confirmed by digestion of pHIR11, and additional subclones were constructed to include restriction fragment boundaries.

Colony hybridization employed the procedures of Maas (32) to lyse the bacteria and bind the DNA to GeneScreen Plus matrix (New England Nuclear/DuPont, Boston, Mass.). Hybridization was performed in 50% formamide in accordance with the matrix manufacturer's recommendations.

Tn*5-gusA1* mutagenesis. λ::Tn*5-gusA1* mutagenesis was performed by the methods of de Bruijn and Lupski (5) as modified by Huang et al. (15). The bacteriophage were propagated in *E. coli* LE392. *E. coli* CC118(pHIR11) cells were grown to the late-log phase in LB broth. The culture was diluted 100-fold and grown at 30°C in LB broth supplemented with 1% (wt/vol) maltose to an optical density at 600 nm of 0.8 (about 10^9 cells per ml). An aliquot (100 μl) of a fresh preparation of λ58::Tn*5-gusA1* phage (a gift of E. R. Signor) was added to 1 ml of the maltose-induced culture and incubated at 30°C for 2 h. The transfected cells were then spread onto LB agar plates containing 100 μg of tetracycline per ml and 250 μg of kanamycin per ml. The plates were incubated at 30°C for 48 h, Kn^r Tc^r colonies were collected, and plasmids were isolated en masse. The plasmids were reintroduced into CC118 cells by electroporation, and CC118 transformants carrying pHIR11::Tn*5-gusA1* were selected on LB agar supplemented with tetracycline and kanamycin. Marker exchange mutagenesis was performed as described by Ruvkin and Ausubel (39) and modified by Huang et al. (15).

Determination of β-glucuronidase (GUS) activity. GUS activity in bacteria grown on agar plates was estimated by a modification of the procedures of Jefferson (19). Two-microliter aliquots of a 1% (wt/vol) 5-bromo-4-chloro-3-indolyl glucuronide solution in dimethylformamide were applied to LB or M63M agar plates by using a grid template, and after drying, each site was inoculated with the indicated bacterium. Plates were incubated at 28°C and scored periodically for the appearance of blue colonies indicative of 5-bromo-4-chloro-3-indolyl glucuronide cleavage.

GUS activity in bacteria grown in liquid medium was determined by fluorometrically monitoring the ability of bacterial lysates to catalyze the hydrolysis of 4-methylumbelliferyl glucuronide (19). Washed bacteria were used to inoculate the indicated medium to 5×10^7 cells per ml. At the designated times, bacteria were harvested, suspended in GUS extraction buffer (50 mM sodium phosphate buffer [pH 7.0], 10 mM β-mercaptoethanol, 10 mM disodium EDTA, 0.1% sodium lauryl sarcosine, 0.1% Triton X-100), and lysed by sonication. GUS activity was determined by measuring the rate of 4-methylumbelliferone accumulation in a reaction mixture consisting of the bacterial lysate diluted 10-fold into GUS extraction buffer supplemented with 1 mM 4-methyl-

umbelliferyl glucuronide. Periodically, 100 μl of the reaction mixture was combined with 900 μl of 0.2 mM Na_2CO_3 to terminate the reaction and methylumbelliferone was estimated by using a Farand System III scanning spectrofluorometer (Valhalla, N.Y.). GUS activity is reported as specific activity (units per milligram of protein). A unit of activity equals 1 pmol of methylumbelliferone formed per min. Protein in the bacterial lysates was estimated by the procedures of Bradford (4). Lysates of *P. syringae* pv. syringae 61, which exhibited no detectable GUS activity, were used as a control in these experiments.

GUS activity in planta was determined in homogenates of tobacco leaves that had been inoculated with a suspension (10^9 cells per ml) of the indicated bacterium. Sections (5 cm^2) of inoculated leaves were excised at the indicated times and homogenized in 100 μl of 10 mM potassium phosphate buffer (pH 7.0). Ten-microliter aliquots of the homogenates were used to estimate bacterial populations by the procedures of Bertoni and Mills (3). GUS extraction buffer (300 μl) was added to 200 μl of the remaining homogenate, and bacteria were lysed by sonication. GUS activity in the homogenates was determined as described above. Leaf tissue infiltrated by *P. syringae* pv. syringae 61 was used as a control. GUS activity was expressed as units per 10^8 bacteria.

Plant fractions. Expressed leaf sap was obtained from excised tobacco leaves by using a pressure bomb (PMS Instrument Co., Corvalis, Oreg.) containing 7 to 15 bars of pressure in the leaf chamber (46). The expressed leaf sap was collected from the cut petiole surface and clarified by centrifugation at 10,000 × *g* for 10 min. Tobacco cell walls (a gift of C. J. Baker) were prepared as described by Baker et al. (1).

Plant reactions. Tobacco (*Nicotiana tabacum* cv. Samsun) leaves were infiltrated with 10^9 bacteria per ml as described by Huang et al. (16) and scored for cell collapse typical of the HR at 24 h.

Osmolarity. Osmotic concentrations of medium were determined by using a Wescor 5500 Vapor Pressure Osmometer in accordance with the manufacturer's instructions.

RESULTS

Construction of *hrp-uidA* fusions. Transcriptional fusions with the functional genes within the *hrp/hrm* gene cluster were constructed by using Tn*5-gusA1*. Initial experiments showed that *P. syringae* pv. syringae 61 produced no detectable GUS activity in culture or in tobacco leaves, thereby allowing use of the *uidA* gene originally isolated from *E. coli* (see reference 19) as a reporter gene. To facilitate mapping of the Tn*5-gusA1* insertions, we established a detailed physical map of the *hrp/hrm* cluster that indicated the presence of 9 *Eco*RV, 11 *Sal*I, 10 *Sma*I, and 7 *Sst*I sites within the cluster, in addition to the previously mapped *Eco*RI, *Hin*dIII, and *Bgl*II sites, and revealed the presence of an additional *Bam*HI site near the originally mapped site (Fig. 1A).

Cosmid pHIR11 was mutagenized with λ::Tn*5-gusA1* in *E. coli* CC118. The pHIR11 derivatives carrying the markers for Tn*5-gusA1* were rapidly screened for insertions by *Eco*RI fragment length polymorphisms. The pHIR11::Tn*5-gusA1* derivatives carrying apparently unique insertions in the *P. syringae* pv. syringae 61-derived DNA were then mobilized into *P. syringae* pv. syringae 61 by triparental mating, and the mutation was transferred into the genome by marker exchange. Tn*5-gusA1* insertions in the pHIR11 derivatives that produced HR^- marker exchange mutants were then carefully mapped, and orientation was determined. Thirteen unique Tn*5-gusA1* insertions were identified (Fig. 1B). Two of these insertions (5165 and 5180) map to a region of pHIR11 not previously known to contain a *hrp* gene.

GUS activity produced by the *hrp*::Tn*5-gusA1* mutants. Because of the possibility that expression of *P. syringae hrp* genes is dependent on a plant factor (cf. reference 10), we initially screened the marker exchange mutants for GUS activity in planta. Early experiments showed that the induction stage for the HR elicited by *P. syringae* pv. syringae 61 in tobacco leaves was 2 to 2.5 h and was not affected by the inoculum concentration up to 10^9 bacteria per ml (data not shown). Assuming that the induction stage represents the time required for expression of the functional genes, we chose 6 h as the time period used in the initial screens in which a significant change in gene expression should be apparent. Ten of the mutants produced detectable GUS activity in tobacco leaves (Fig. 1B). Three produced only trace amounts of GUS activity in culture or when inoculated into tobacco and were later shown to be oriented opposite to the deduced direction of transcription for the mutated locus.

Deduced transcriptional organization of the *P. syringae* pv. syringae *hrp* cluster. On the basis of the orientation of the Tn*5*::*gusA1* insertions and their relative GUS activities, the deduced direction of transcription for the Tn*phoA*-defined *hrp* complementation groups was established. The deduced direction of transcription for *hrp* complementation groups I, II, III, V, VI, and VII was determined to be rightward as drawn, whereas that of groups VIII, X, XI, and XIII was leftward (Fig. 1B). An analysis of the bioluminescence produced by a promotorless *lux-kan* cassette (43) inserted in both orientations in the leftmost *Bgl*II site within the cluster showed that the direction of transcription for *hrpIV* is also rightward. GUS activities of pHIR11BB8::Tn*5-gusA1* derivatives that mapped to *hrp* complementation groups I, II, III, and IV are consistent with the proposed transcriptional orientation of these groups (data not shown). We were unable to isolate Tn*5-gusA1* insertions in *hrmA* or *hrpIX*.

To confirm the mapped location of the pHIR11::Tn*5-gusA1* insertions and further characterize the transcriptional organization of the cluster, we constructed merodiploids in which the pHIR11::Tn*5-gusA1* derivatives were mobilized into a representative set of *P. syringae* pv. syringae 61 *hrp*::Tn*phoA* marker exchange mutants previously characterized by Huang et al. (15). The pHIR11::Tn*5-gusA1* derivatives failed to complement the HR^- *P. syringae* pv. syringae 61 mutants containing Tn*phoA* insertions that mapped to the same complementation group (sensu reference 15) and retained an HR^- phenotype in tobacco (Fig. 1C). The Tn*5-gusA1* insertions in pHIR11-5180 and pHIR11-5165 appeared to be located in a separate complementation group, henceforth designated *hrpI*.

The genetic complexity of this cluster relative to the *hrp* cluster present in *P. syringae* pv. phaseolicola (38) raised the possibility that Tn*phoA* causes nonpolar mutations in *P. syringae* pv. syringae 61 (15). The complementation groups defined by Tn*phoA* mutagenesis, therefore, could represent translational units. Several of the Tn*5-gusA1* insertions had a polar effect on the ability to complement chromosomal mutations in the contiguous complementation groups apparently located transcriptionally downstream of the mutated locus. A Tn*5-gusA1* insertion into *hrpIII*, pHIR11-5112, failed to complement Tn*phoA* mutations in *hrpIV* (Fig. 1C) but retained the ability to complement mutations in *hrpII* and *hrpV*. Similar results were obtained for complementation groups V to VII and XI-XII. Tn*5-gusA1* insertions that map to the initial *hrp* complementation group within an apparent

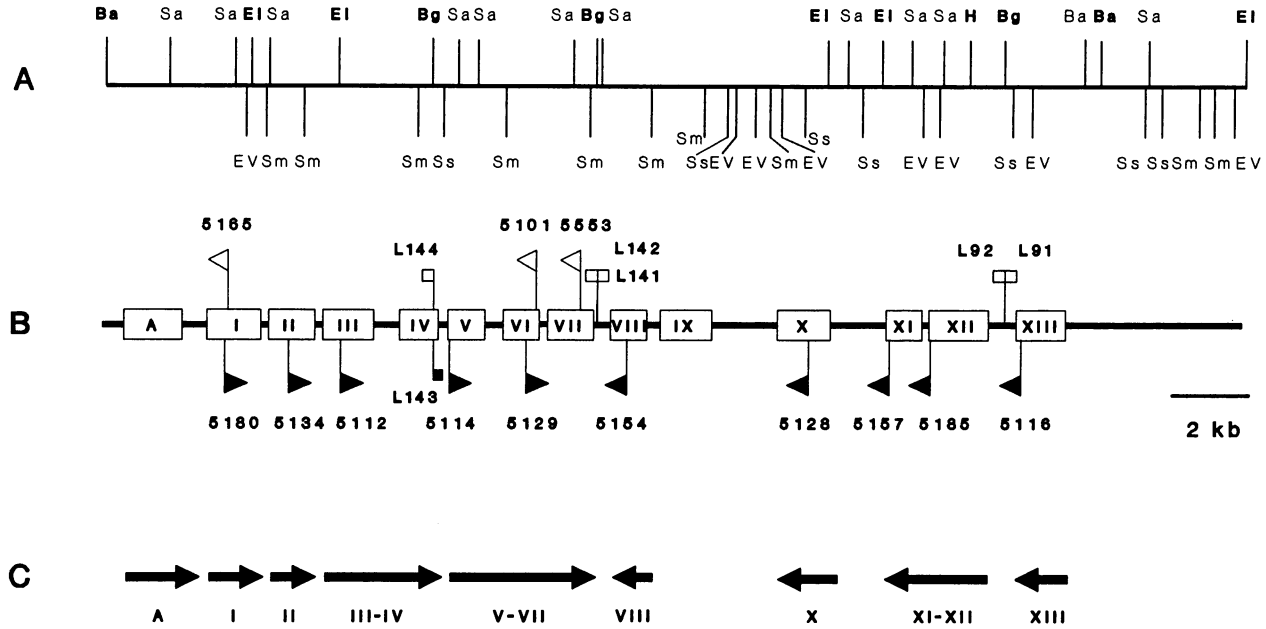

FIG. 1. Physical map, map of Tn*5-gusA1* insertions, and transcriptional organization of the *hrp* gene cluster of *P. syringae* pv. syringae 61. (A) Restriction map of the *hrp/hrm* region of *P. syringae* pv. syringae 61. Abbreviations: Ba, *Bam*HI; Bg, *Bgl*II; EI, *Eco*RI; EV, *Eco*RV; H, *Hin*dIII; Sa, *Sal*I; Sm, *Sma*I; Ss, *Sst*I. Previously mapped restriction sites are in boldface. (B) Tn*5-gusA1* and *lux-nptI* insertion sites. Boxes labeled with roman numbers II to XIII indicate the *hrp* complementation groups described by Huang et al. (15). The *hrmA* locus (15) is designated by the letter A. The designations chosen by Huang et al. (15) were retained to avoid confusion with the designations used for the partially homologous *P. syringae* pv. phaseolicola *hrp* cluster (38). The positions and orientation of the mapped Tn*5-gusA1* insertions are showed by triangular flags. At least two apparent siblings were identified for each insertions shown that mapped to within 0.2 kb of the indicated insertion. The square flags indicate the insertion sites for a *lux-nptI* cassette isolated as a 7.5-kb *Bam*HI fragment from Tn*4431* (43) and ligated in both orientations into the *Bgl*II sites shown above in panel A. The resulting insertions were transferred into the *P. syringae* pv. syringae 61 genome by marker exchange. Filled flags are those insertions producing detectable reporter activity in planta. Marker exchange mutants L141, L142, L91, and L92 retained an HR^+ phenotype. (C) Apparent transcriptional organization of the *hrp/hrm* cluster. Complementation groups were characterized in *P. syringae* pv. syringae 61 *hrp*::Tn*phoA* merodiploids carrying pHIR11::Tn*5-gusA1* derivatives. The pHIR11::Tn*5-gusA1* derivatives defined in panel B were mobilized by triparental matings into *P. syringae* pv. syringae 61 *hrp* mutants with mutations in the same or adjacent complementation groups, and the transconjugants were screened for the ability to elicit the HR in tobacco. *P. syringae* pv. syringae 61 *hrp*::Tn*phoA* mutants employed (locus): 2070 (*hrmA*), 2074 (*hrpII*), 2075 (*hrpIII*), 2077 (*hrpIV*), 2081 (*hrpV*), 2084 (*hrpVI*), 2086 (*hrpVII*), 2087 (*hrpVIII*), 2088 (*hrpIX*), 2089 (*hrpX*), 2091 (*hrpXI*), 2092 (*hrpXII*), and 2094 (*hrpXIII*). The Tn*5-gusA1*-defined complementation groups are indicated by the bars and are termed apparent transcriptional units. Arrowheads indicate the deduced directions of transcription.

transcriptional unit failed to complement any Tn*phoA* insertions within that unit; Tn*5-gusA1* insertions in downstream complementation groups retained the ability to complement insertions in upstream complementation groups. This suggests that *hrpIII-IV*, *hrpV-VII*, and *hrpXI-XII* represent multicistronic transcriptional units. The 13 *hrp* complementation groups appear to be organized into eight apparent transcriptional units.

Temporal expression of the *hrp-uidA* fusions in planta. Seven of the apparent transcriptional units (I, II, III-IV, V-VII, VIII, X, and XI-XII) exhibited enhanced expression in tobacco leaves (Table 2). GUS activity in mutants carrying expressed Tn*5-gusA1* fusions in these apparent transcriptional units increased two- to ninefold during the initial 6 h of the interaction. A fusion (5116) in the remaining unit produced detectable GUS activity within 15 min of inoculation of tobacco leaves, produced high levels of GUS activity during growth in KB medium, and was assumed to be constitutively expressed.

Strain 61-5112, which carries an insertion that maps to the *hrpIII* complementation groups, was chosen for further study. Enhanced GUS activity in tobacco tissue inoculated with 61-5112 was detected beginning 2 h after inoculation of

TABLE 2. Expression of selected *hrp*::Tn*5-gusA1* derivatives in tobacco leaves

Mutant	ATU[a]	Mean GUS activity[b] ± SEM (U/10^8 CFU)		Induction[c]
		Initially	After 6 h	
61-5180	I	44 ± 6	332 ± 55	6.5
61-5134	II	59 ± 23	218 ± 120	2.7
61-5112	III–IV	9 ± 3	94 ± 12	9.4
61-5114	V–VII	9 ± 2	42 ± 10	3.7
61-5154	VIII	1,510 ± 681	11,289 ± 1,676	6.5
61-5128	X	45 ± 10	199 ± 71	3.4
61-5157	XI–XII	21 ± 5	57 ± 10	1.7
61-5180	XIII	64 ± 6	47 ± 6	0

[a] ATJ, apparent transcriptional unit.

[b] *P. syringae* pv. syringae mutants were grown in KB broth until the culture was in mid-logarithmic growth. Tobacco leaves were inoculated with the indicated bacterium suspended in 10 mM potassium phosphate buffer (pH 7.0) at 10^9 cells per ml. At the indicated times, leaf panels were excised and GUS activity and bacterial populations were determined in leaf samples as described in Materials and Methods.

[c] Fold increase in GUS activity was determined as follows: (activity at 6h/initial activity) − 1.

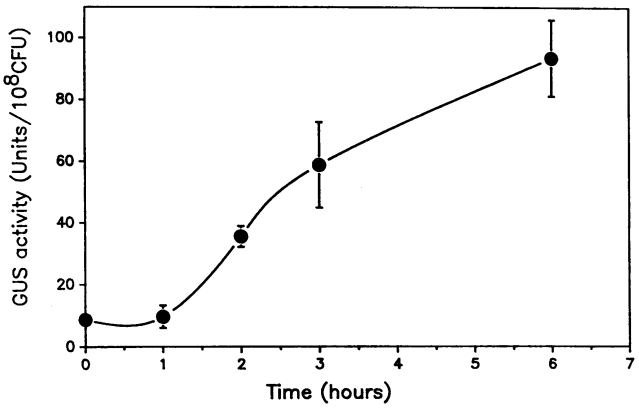

FIG. 2. Induction of *hrpIII* expression following inoculation of tobacco leaves. A suspension of *P. syringae* pv. syringae 61-5112 (10^9 cells per ml) was inoculated into tobacco leaves, and GUS activities and bacterial populations were measured at the indicated times as described in Materials and Methods. The values presented are means of three replicates. Error bars indicate standard deviations. Similar results were obtained in at least two additional experiments.

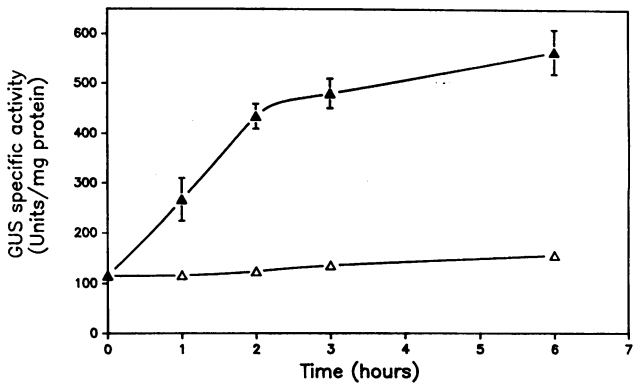

FIG. 3. Induction of *hrpIII* expression in minimal-salts medium. Washed *P. syringae* pv. syringae 61-5112 cells were used to inoculate M63M (▲) or KB (△) medium to 5×10^7 cells per ml. At the indicated times, bacteria were harvested and GUS specific activity was determined in lysates as described in Materials and Methods. Total protein was employed as the reference standard to avoid artifactual results due to differences in cell volume between the two media. The values reported represent the means of three replicates. Error bars indicate standard deviations.

tobacco leaves (Fig. 2). During the initial hour of the interaction, GUS activities were equivalent to those observed in KB medium. By 24 h, a 52-fold enhancement in GUS activity was observed.

During the course of these experiments, we observed that populations of *P. syringae* pv. syringae 61-5112 detected in tobacco leaves decreased approximately 30% during the initial 6 h of the interaction and were only 9% of the initial level by 24 h (data not shown). In contrast, GUS activity in tobacco leaves infiltrated with bacterial lysates exhibited a halflife of greater than 9 h. The stability of GUS relative to the bacteria, therefore, may inflate the levels of induction calculated to occur in planta during later stages of the interaction. Henceforth, 3 h of incubation was used to test the influence of environmental factors on expression of the *hrp-uidA* fusions.

Expression of the *hrpIII-uidA* fusion in culture. The seven representative *hrp-uidA* fusions that exhibited enhanced expression in planta also exhibited enhanced GUS activity when grown on a minimal-salts medium, such as M63M agar. Low levels of GUS activity were detected in bacteria maintained in KB medium, irrespective of the growth phase of the culture. The specific activity of GUS increased four- to sixfold within 3 h of transfer of *P. syringae* pv. syringae 61-5112 from KB medium to M63M medium (Fig. 3). Carbon source had little effect on the expression observed in either medium. Similar levels of expression were detected when glucose, glycerol, or succinate was substituted for the mannitol in M63M medium or when the glycerol in KB medium was replaced with mannitol, glucose, or succinate. Acidification of either medium did not stimulate expression of the *hrp*III-*uidA* fusion in *P. syringae* pv. syringae 61-5112.

Role of a plant factor in induction of *hrpIII* expression. The enhancement of *hrpIII* expression observed in tobacco leaves after 3 h (six- to sevenfold) was slightly higher than that observed in minimal-salts medium (four- to sixfold). To explore the possibility that a plant factor affects the expression of *P. syringae* pv. syringae 61 *hrp* genes, we monitored expression of the *hrpIII*::Tn*5-gusA1* fusion (5112) in expressed leaf sap. Expressed leaf sap consists primarily of xylem fluids which are representative of the apoplastic fluids that wet plant cell walls in the leaf mesophyll (25) and therefore should contain any soluble effectors that the bacteria would be exposed to in planta. Induction of GUS activity detected when *P. syringae* pv. syringae 61-5112 was cultured in expressed leaf sap or leaf sap supplemented with isolated plant cell walls was equivalent to that observed in M63M medium (Table 3). Addition of tobacco leaf homogenates to M63M medium containing *P. syringae* pv. syringae 61-5112 did not further enhance apparent expression of *hrpIII* (data not shown).

Repression of *hrpIII* expression by complex nitrogen sources. To determine whether the peptone present in KB medium was repressive to expression of *hrpIII*, induction of GUS activity in *P. syringae* pv. syringae 61-5112 was monitored in M63M medium supplemented with peptone. Addition of as little as 0.0285% peptone (equivalent to 2 mM N equivalents) to the medium caused a substantial decrease in the apparent induction of *hrpIII* in strain 61-5112 (Fig. 4). When the peptone concentration was increased to 0.285% (20 mM N equivalents) or higher, the specific activity of GUS was equivalent to that observed in KB medium. Similar results were obtained when proteose peptone was replaced by a casein hydrolysate (data not shown).

The effect of peptone could not be attributed to nitrogen metabolism or medium osmolarity. Addition of excess ammonia to the medium had little effect on expression of *hrpIII* (Fig. 4). The specific activity of GUS in bacteria grown in

TABLE 3. Effects of plant extracts on *hrpIII* expression

Growth condition[a]	Mean Gus activity[b] ± SEM (U/mg of protein) after 3 h	Induction[c]
M63M	222 ± 4	6.2
Expressed leaf sap	152 ± 9	4.2
Expressed leaf sap–isolated tobacco cell walls	131 ± 3	3.6

[a] *P. syringae* pv. syringae 61-5112 was grown in KB broth until the culture was in mid-logarithmic growth. Cells were harvested and suspended in 1 ml of the indicated medium to 5×10^7/ml. GUS specific activity was determined in bacteria harvested at the indicated times as described in Materials and Methods.

[b] In each case, the initial GUS activity was 36 ± 5 U/mg of protein.

[c] Fold increase in GUS activity is shown. The GUS activity of bacteria maintained in KB medium remained within 20% of the initial activity.

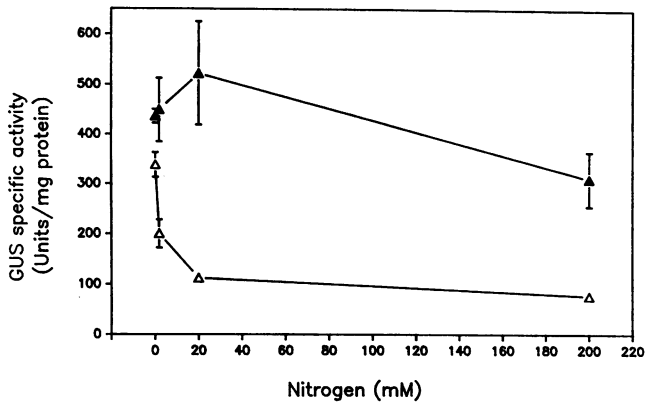

FIG. 4. Repression of *hrpIII* expression by peptone. Washed *P. syringae* pv. syringae 61-5112 cells were suspended to 5×10^7/ml in modified M63M medium in which peptone (△) or ammonium sulfate (▲) at the indicated concentrations was provided as the sole nitrogen source. After 3 h, the bacteria were harvested and GUS specific activity was determined in lysates as described in Materials and Methods. Each value represents the mean of three independent replicates. Error bars indicate standard deviations.

M63M medium supplemented with 100 mM ammonia was equivalent to that of bacteria grown in M63M. Similar results were obtained when glutamine was used as the nitrogen source (data not shown). No single amino acid (20 mM), when added to M63M, repressed *hrpIII* expression (data not shown). The osmolarities of the media used to obtain the data for Fig. 4 were indistinguishable for equivalent N concentrations (±2%). Media of higher osmolarity, however, were repressive to *hrpIII* expression. Addition of 0.5 M NaCl, 0.5 M KCl, or 0.33 M K_2SO_4 to M63M medium reduced the GUS activity to less than half of that observed in KB medium but substantially reduced the growth rate of the bacteria as well. Addition of a metabolizable solute, such as mannitol, inhibited *hrpIII* expression at concentrations exceeding 400 mM (data not shown).

DISCUSSION

Elucidation of the molecules that function in plant-pathogen recognition during incompatible interactions has been delayed in part by the apparent requirement for bacterial gene expression during the induction stage of the HR. It has been previously established that a set of *hrp/hrm* genes isolated from *P. syringae* pv. syringae 61 is sufficient to enable nonpathogenic bacteria to elicit the HR (11, 15, 16, 17a) and suggested that these genes are environmentally regulated (17, 53). We can now confirm that expression of most of the *P. syringae* pv. syringae 61 *hrp* complementation groups defined previously (15) is repressed during growth in rich medium and increases following inoculation of a nonhost plant species within the time frame of the HR induction stage. Enhanced expression from these genes, as indicated by accumulation of GUS, was observed within 2 h after infiltration of tobacco leaves. The work of Heu et al. (11, 17a) and Huang et al. (15) suggests that the *hrp/hrm* gene cluster has the capacity to produce the primary factor or activity that initiates the HR. The induction stage of the HR, therefore, may represent the time required for the *hrp/hrm* genes to be expressed and their products to accumulate.

Studies on other plant-pathogenic (*Agrobacterium* and *Pseudomonas* spp.) or -symbiotic (*Rhizobium*, *Bradyrhizobium*, and *Sinorhizobia* spp.) bacteria have implicated plant factors (e.g., phenolic compounds and flavonoids) in environmental regulation of the pathogenicity and symbiosis genes (see references 30, 34, 35, and 48). Several *P. syringae* pv. phaseolicola *hrp* genes are rapidly induced in leaves of susceptible bean plants (27); this has been attributed to an unidentified plant effector (9, 38). Our results, which were obtained with a different host range variant of *P. syringae*, focused on the early phases of the interaction with a nonhost plant species, and utilized a different reporter gene system (GUS versus ice nucleation), are inconsistent with the involvement of a plant factor in the regulation of *P. syringae* pv. syringae 61 *hrp* genes during the interaction with tobacco. Similar levels of expression were detected when the bacteria were transferred into M63M minimal-salts medium and during early stages of the interaction with tobacco cells. Plant extracts failed to stimulate *hrpIII* expression over that observed in M63M medium. Instead, our results agree with those of Yucel et al. (53), which suggested that nutritional conditions affect the expression of genes induced during the induction stage of the HR. Peptone or casein hydrolysate, common constituents of the culture media used to grow *P. syringae* strains, can repress the expression of most *hrp* genes at concentrations >50-fold more dilute than that present in commonly used culture medium (KB or nutrient broth). Inoculation into the plant or transfer into minimal-salts medium appears to initiate the induction process by altering the nutritional conditions. It is interesting that *phoA* fusions with *hrpIV* and *hrpX* that produce membrane-spanning or exported products could be detected only when the bacteria were grown in minimal-salts medium (15). Growth of the bacteria in minimal-salts medium abolishes or significantly shortens the induction stage for the plant response (18, 26, 51, 53). These observations, however, do not exclude the possibility that plant factors influence expression of the *P. syringae* pv. syringae 61 *hrp/hrm* genes during the interaction with other plant species and that other host range variants of *P. syringae* utilize such signals (cf. reference 38).

The nature of the peptone repression of *hrp* expression in *P. syringae* pv. syringae 61 remains elusive. It does not appear to be due to the catabolite repression previously reported to affect the expression of a plasmid-borne *avrB'-lacZ* fusion in *P. syringae* pv. glycinea race 0 (18). In their study, dicarboxylic acids, such as succinate, were nearly as effective as peptone in repressing accumulation of β-galactosidase in *P. syringae* pv. glycinea race 0 carrying the *avrB'-lacZ* fusion. Glycerol was also repressive when provided as the sole carbon source. In our work, glycerol and succinate were as effective as mannitol in supporting induction of the *hrpIII* locus in minimal-salts medium. It has also been reported that high osmotic pressure can repress the expression of several *hrp* genes in *P. syringae* pv. phaseolicola (9, 38). High osmotic pressure also inhibited induction of *hrpIII* in *P. syringae* pv. syringae 61, but at levels substantially higher than that of KB medium or peptone-supplemented M63M medium. Relatively low levels of peptone were sufficient to suppress GUS accumulation in strain 61-5112. Addition of other solutes at equivalent osmolarities had little effect on *hrpIII* induction. The effect could not be attributed to a specific component of peptone; casein hydrolysate was equally repressive to *hrpIII* expression. In *Erwinia amylovora*, regulation of several *hrp* genes has been linked to nitrogen metabolism (2) but inorganic nitrogen sources, such as ammonium sulfate, as well as individual amino acids, had little effect on induction of *hrpIII* in *P. syringae* pv. syringae 61-5112, suggesting that the *P. syrin-*

gae pv. syringae *hrp* genes are regulated via a different mechanism. It may be that expression of *P. syringae* pv. syringae 61 *hrp* genes is controlled, in part, by stringent conditions, as originally suggested by Yucel et al. (53), or by a combination of amino acids.

In these experiments, we worked with *P. syringae* pv. syringae 61 *hrp*::Tn*5-gusA1* mutants that were unable to elicit a plant response. Results similar to those described above were obtained with *P. syringae* pv. syringae 61 merodiploids carrying pHIR11::Tn*5-gusA1* derivatives that retained an HR^+ phenotype. Those *hrp-uidA* fusions that exhibited enhanced expression in minimal-salts medium when chromosomal also exhibited enhanced expression in minimal-salts medium in the merodiploids. Levels of expression detected, however, were 50 to 75 times higher than the chromosomal equivalents and were affected by the level of antibiotics included in the medium. These observations suggest that plasmid copy number, repressor titration, or vector-directed expression may influence *hrp* gene expression in plasmid-borne constructs. Levels of expression from the plasmid-borne *hrp-uidA* fusions in bacteria grown in KB medium were greater than that observed from chromosomal fusions following induction in minimal-salts medium or in planta. This apparent high-level basal expression from the plasmid-borne constructs may explain the inability to detect an induction stage in strains harboring pHIR11 (51) and could explain the rapid responses induced by strains carrying this plasmid (11, 16, 17a). To avoid these potential artifacts, subsequent experiments employed chromosomal *hrp*::Tn*5-gusA1* mutants of *P. syringae* pv. syringae 61. Keppler et al. (22a) showed that the initial responses of plant cells to inoculated bacteria may be similar, irrespective of their pathogenicity. This suggests that similar conditions exist in plant tissue, irrespective of the activity of the *hrp* cluster.

The *P. syringae* pv. syringae *hrp/hrm* cluster appears to be organized as at least eight apparent transcriptional units, at least seven of which exhibit enhanced expression in planta. The deduced transcriptional orientation of *hrpI*, *hrpII*, *hrpIII-IV*, and *hrpV-VII* is rightward, whereas that of *hrpVIII*, *hrpX*, *hrpXI-XII*, and *hrpXIII* is leftward. The polarities of Tn*5-gusA1* mutations in complementation analyses are consistent with the deduced organization. This general organization appears to be similar to that of the partially homologous *P. syringae* pv. phaseolicola NPS3121 *hrp* cluster (15, 28, 38), although the homologous loci within each cluster have not been established. Sequence analysis has identified large open reading frames at the mapped locations of *hrp* complementation groups I (13), II (31), III (52), IV (14), and X (14) whose orientation agrees with the deduced transcriptional organization of the cluster. The nucleotide sequence of the *hrmA* locus and primer extension data indicate that its transcriptional orientation is also rightward (12; Fig. 1C). It has not been established whether *hrmA* is a transcriptional unit separate from *hrpI*. Unfortunately, no Tn*5-gusA1* insertions were identified in *hrpIX*. Characterization of the transcriptional orientation of *hrpIX* requires further study. If the *hrp* cluster of *P. syringae* pv. phaseolicola (15) is colinear with the partially homologous *P. syringae* pv. syringae 61 *hrp/hrm* cluster, then *hrpIX* is predicted to be transcriptionally linked with *hrpVIII* (cf. reference 38).

Since the initial description of *hrp* genes in *P. syringae* pv. phaseolicola (29, 36), homologous or analogous genes have been described in other plant-pathogenic bacteria (e.g., several other *P. syringae* pathovars, *P. solanacearum*, *Xanthomonas campestris*, and *E. amylovora*; see reference 47). *hrp* genes play an essential role in the behavior of these bacteria in both susceptible and resistant plant species (29, 36, 47). It appears that enhanced expression of *hrp* genes is observed during compatible interactions in which disease develops (9, 27, 38) and incompatible interactions in which the HR develops. The environmental stimuli that affect the expression of *hrp* or *avr* genes in each of the host range variants of *P. syringae* studied (*P. syringae* pv. glycinea race 0 [18], *P. syringae* pv. phaseolicola NPS3121 [9, 38], and *P. syringae* pv. syringae 61 [reported here]) appear to differ. The *hrp/hrm* gene cluster appears to be sufficient to produce an HR-eliciting signal in nonpathogenic and pathogenic bacteria, expression of these genes is induced within the time frame of the induction stage of the HR, the culture conditions that affect the duration of the induction stage also affect the expression of these *hrp* genes, elevated expression from a cloned *hrp/hrm* gene cluster diminishes the duration of the induction stage, and these genes appear to have the capacity to interface with the external medium (see references 15 and 26). Differential expression of these *hrp* genes in response to distinct environmental signals or the differential activity of Hrp products during the interaction with susceptible or resistant plant hosts, therefore, may play an important role in determining the host range of these bacteria.

ACKNOWLEDGMENTS

This research was funded in part by National Science Foundation grant DCB8716967 and a fellowship provided to Y.X. by the Center for Agricultural Biotechnology of the Maryland Biotechnology Institute.

We extend our gratitude to H. Sze and S. Wolniak for use of their spectrofluorometer, to E. Signor for providing Tn*5-gusA1*, and to I. Forseth for his assistance with preparation of expressed leaf extracts.

REFERENCES

1. **Baker, C. J., N. Mock, M. M. Atkinson, and S. W. Hutcheson.** 1990. Inhibition of the hypersensitive response in tobacco by pectate lyase digests of cell wall and of polygalacturonic acid. Physiol. Mol. Plant Pathol. **37**:155–167.
2. **Beer, S. V., D. W. Bauer, X. H. Jiang, R. J. Laby, B. J. Sneath, Z.-M. Wei, D. A. Wilcox, and C. H. Zumoff.** 1991. The *hrp* gene cluster of *Erwinia amylovora*, p. 53–60. *In* H. Hennecke and D. P. S. Verma (ed.), Advances in molecular genetics of plant-microbe interactions, vol. 1. Kluwer Academic Publishers, Dordrecht, The Netherlands.
3. **Bertoni, G., and D. Mills.** 1987. A simple method to monitor growth of bacterial populations in leaf tissue. Phytopathology **77**:832–835.
4. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
5. **de Bruijn, F. J., and J. R. Lupski.** 1984. The use of transposon Tn*5* mutagenesis in the rapid generation of correlated physical and genetic maps of DNA segments cloned into multicopy plasmids—a review. Gene **27**:131–149.
6. **Ditta, G., S. Stanfield, D. Corbin, and D. R. Helinski.** 1980. Broad host range DNA cloning system for gram negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA **77**:7347–7351.
7. **Dixon, R. A., and C. Lamb.** 1990. Molecular communication in interactions between plant and microbial pathogens. Annu. Rev. Plant Physiol. **41**:339–367.
8. **Dower, W. J., J. F. Miller, and C. W. Ragsdale.** 1988. High efficiency transformation of *Escherichia coli* by high voltage electroporation. Nucleic Acids Res. **16**:6127–6145.
9. **Fellay, R., L. G. Rahme, M. N. Mindrinos, R. D. Frederick, A. Pisi, and N. J. Panopoulos.** 1991. Genes and signals controlling the *Pseudomonas syringae* pv. *phaseolicola*-plant interaction, p. 45–52. *In* H. Hennecke and D. P. S. Verma (ed.), Advances in molecular genetics of plant-microbe interactions, vol. 1. Kluwer Academic Publishers, Dordrecht, The Netherlands.
10. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli*

with plasmids. J. Mol. Biol. **166**:557–580.
11. **Heu, S., and S. W. Hutcheson.** 1991. *Pseudomonas syringae* pv. syringae *hrp/hrm* genes encode avirulence functions in *P. syringae* pv. *glycinea* race 4. Phytopathology **81**:702–703.
12. **Heu, S., and S. W. Hutcheson.** 1991. Molecular characterization of the *Pseudomonas syringae* pv. *syringae* 61 *hrm*A locus. Phytopathology **81**:1245.
13. **Heu, S., Y. Lu, and S. W. Hutcheson.** Unpublished data.
14. **Huang, H.-C., and A. Collmer.** Unpublished data.
15. **Huang, H.-C., S. W. Hutcheson, and A. Collmer.** 1991. Characterization of the *hrp* cluster from *Pseudomonas syringae* pv. *syringae* 61 and Tn*phoA* tagging of genes encoding exported or membrane-spanning Hrp proteins. Mol. Plant-Microbe Interact. **4**:469–476.
16. **Huang, H.-C., R. Schuurink, T. P. Denny, M. M. Atkinson, C. J. Baker, I. Yucel, S. W. Hutcheson, and A. Collmer.** 1988. Molecular cloning of a *Pseudomonas syringae* pv. *syringae* gene cluster that enables *Pseudomonas fluorescens* to elicit the hypersensitive response in tobacco plants. J. Bacteriol. **170**:4748–4756.
17. **Hutcheson, S. W., C. J. Baker, and A. Collmer.** 1989. Elicitation of the hypersensitive response by *Pseudomonas syringae*. Physiol. Plant. **76**:155–163.
17a. **Hutcheson, S. W., S. Heu, H. C. Huang, T.-H. Li, Y. Lu, and Y. Xiao.** The *hrp* genes of *Pseudomonas syringae*: an evaluation of their role in determining nonhost resistance. *In* D. Bills and S. D. King (ed.), Biotechnology and plant protection: bacterial pathogenesis and plant resistance, in press. Butterworths, London.
18. **Huynh, T., D. Dahlbeck, and B. J. Staskawicz.** 1989. Bacterial blight of soybean: regulation of a pathogen gene determining host cultivar specificity. Science **245**:1374–1377.
19. **Jefferson, R. A.** 1987. Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol. Biol. Rep. **5**:387–405.
20. **Keen, N. T.** 1990. Gene for gene complementarity in plant-pathogen interactions. Annu. Rev. Genet. **24**:447–463.
21. **Keen, N. T., and M. J. Holliday.** 1982. Recognition of bacterial pathogens by plants, p. 179–220. *In* M. S. Mount and G. H. Lacy (ed.), Phytopathogenic prokaryotes, vol. 2. Academic Press, Inc., New York.
22. **Keen, N. T., and B. J. Staskawicz.** 1988. Host range determinants in plant pathogens and symbionts. Annu. Rev. Microbiol. **42**:421–440.
22a. **Keppler, L. D., C. J. Baker, and M. M. Atkinson.** 1989. Active oxygen production during a bacteria-induced hypersensitive reaction in tobacco suspension cells. Phytopathology **79**:974–978.
23. **King, E. O., M. K. Ward, and D. E. Raney.** 1954. Two simple media for the demonstration of pyocyanin and fluorescein. J. Lab. Clin. Med. **44**:301–307.
24. **Klement, Z.** 1982. Hypersensitivity, p. 149–177. *In* M. S. Mount and G. H. Lacy (ed.), Phytopathogenic prokaryotes, vol. 2. Academic Press, Inc., New York.
25. **Kramer, P. J.** 1983. Water relations of plants. Academic Press, Inc., New York.
26. **Li, T.-H., S. A. Benson, and S. W. Hutcheson.** 1992. Phenotypic expression of the *Pseudomonas syringae* pv. syringae 61 *hrp/hrm* gene cluster in *Escherichia coli* MC4100 requires a functional porin. J. Bacteriol. **174**:1742–1749.
27. **Lindgren, P. B., R. D. Frederick, A. G. Govindarajin, N. J. Panopoulos, B. J. Staskawicz, and S. E. Lindow.** 1989. An ice nucleation reporter gene system: identification of inducible pathogenicity genes in *Pseudomonas syringae* pv. *phaseolicola*. EMBO J. **8**:2990–3001.
28. **Lindgren, P. B., N. J. Panopoulos, B. J. Staskawicz, and D. Dahlbeck.** 1988. Genes required for pathogenicity and hypersensitivity are conserved and interchangeable among pathovars of *Pseudomonas syringae*. Mol. Gen. Genet. **211**:499–506.
29. **Lindgren, P. B., R. C. Peet, and N. J. Panopoulos.** 1986. Gene cluster of *Pseudomonas syringae* pv. "*phaseolicola*" controls pathogenicity of bean plants and hypersensitivity on nonhost plants. J. Bacteriol. **168**:512–522.
30. **Long, S. R.** 1989. *Rhizobium* genetics. Annu. Rev. Genet. **23**:483–506.
31. **Lu, Y., and S. W. Hutcheson.** Unpublished data.
32. **Maas, R.** 1983. An improved colony hybridization method with significantly increased sensitivity for detection of single genes. Plasmid **10**:296–298.
33. **Manoil, C., and J. Beckwith.** 1985. Tn*phoA*: a transposon probe for protein export signals. Proc. Natl. Acad. Sci. USA **82**:8129–8133.
34. **Mo, Y.-Y., and D. C. Gross.** 1991. Plant signal molecules activate the *syrB* gene, which is required for syringomycin production by *Pseudomonas syringae* pv. syringae. J. Bacteriol. **173**:5784–5792.
35. **Nester, E. W., and M. P. Gordon.** 1991. Molecular strategies in the interaction between *Agrobacterium* and its hosts, p. 3–9. *In* H. Hennecke and D. P. S. Verma (ed.), Advances in molecular genetics of plant-microbe interactions, vol. 1. Kluwer Academic Publishers, Dordrecht, The Netherlands.
36. **Niepold, F., D. Anderson, and D. Mills.** 1985. Cloning determinants of pathogenesis from *Pseudomonas syringae* pathovar *syringae*. Proc. Natl. Acad. Sci. USA **82**:406–410.
37. **Perbal, B.** 1988. A practical guide to molecular cloning, 2nd ed. John Wiley & Sons, Inc., New York.
38. **Rahme, L. G., M. N. Mindrinos, and N. J. Panopoulos.** 1991. Genetic and transcriptional organization of the *hrp* cluster of *Pseudomonas syringae* pv. phaseolicola. J. Bacteriol. **173**:575–586.
39. **Ruvkin, G. B., and F. M. Ausubel.** 1981. A general method for site-directed mutagenesis in prokaryotes. Nature (London) **289**: 85–88.
40. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
41. **Sasser, M.** 1982. Inhibition by antibacterial compounds of the hypersensitive reaction induced by *Pseudomonas pisi* in tobacco. Phytopathology **72**:1513–1517.
42. **Sharma, S. B., and E. R. Signor.** 1990. Temporal and spatial regulation of the symbiotic genes of *Rhizobium meliloti* in planta revealed by transposon Tn*5-gus*A. Genes Dev. **4**:344–356.
43. **Shaw, J. J., and C. I. Kado.** 1986. Development of a *Vibrio* bioluminescence gene-set to monitor phytopathogenic bacteria during the ongoing disease process in a non-disruptive manner. Bio/Technology **4**:560–564.
44. **Silhavy, T. J., M. L. Berman, and L. W. Enquist.** 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
45. **Staskawicz, B., D. Dahlbeck, N. Keen, and C. Napoli.** 1987. Molecular characterization of cloned avirulence genes from race 0 and race 1 of *Pseudomonas syringae* pv. *glycinea*. J. Bacteriol. **169**:5789–5794.
46. **Tyree, M. T., and H. T. Hammel.** 1972. The measurement of turgor pressure and the water relations of plants by the pressure bomb technique. J. Exp. Bot. **23**:267–282.
47. **Willis, K., J. J. Rich, and E. M. Hrabak.** 1990. The *hrp* genes of phytopathogenic bacteria. Mol. Plant-Microbe Interact. **4**:132–138.
48. **Winins, S.** 1990. Transcriptional induction of an *Agrobacterium* regulatory gene at tandem promotors by plant-released phenolic compounds, phosphate starvation, and acidic growth media. J. Bacteriol. **172**:2433–2438.
49. **Xiao, Y., and S. W. Hutcheson.** 1991. Environmental regulation of *Pseudomonas syringae* pv. *syringae* 61 *hrp* genes. Phytopathology **81**:705.
50. **Xiao, Y., and S. W. Hutcheson.** 1991. Transcriptional organization and expression of the *Pseudomonas syringae* pv. *syringae* 61 *hrp* gene cluster. Phytopathology **81**:1245–1246.
51. **Xiao, Y., and S. W. Hutcheson.** Unpublished data.
52. **Xiao, Y., Y. Lu, and S. W. Hutcheson.** Unpublished data.
53. **Yucel, I., Y. Xiao, and S. W. Hutcheson.** 1989. Influence of *Pseudomonas syringae* culture conditions on initiation of the hypersensitive response of cultured tobacco cells. Appl. Environ. Microbiol. **55**:1724–1729.