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Plant and Environmental Sensory Signals Control the Expression of *hrp* Genes in *Pseudomonas syringae* pv. phaseolicola

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The *hrp* genes of *Pseudomonas syringae* pv. phaseolicola control the development of primary disease symptoms in bean plants and the elicitation of the hypersensitive response in resistant plants. We examined the expression of the seven operons located in the 22-kb *hrp* cluster (L. G. Rahme, M. N. Mindrinos, and N. J. Panopoulos, J. Bacteriol. 173:575–586, 1991) in planta and in vitro under different physiological and nutritional conditions by using chromosomally located *hrp::inaZ* reporter fusions. We show that (i) a plant signal(s) is specifically required for the induction of the seven *hrp* operons, during both compatible and incompatible interactions; (ii) *hrpL* and *hrpRS* are regulated by different mechanisms in planta and in vitro; and (iii) expression of individual *hrp* loci is differentially affected by pH, osmotic strength, and type of carbon source: *hrpAB*, *hrpC*, and *hrpD* were downregulated similarly by osmolarity, pH, and certain carbon sources; *hrpE* expression was affected strongly by pH and carbon substrate and slightly by osmolarity; and *hrpF* was not substantially affected by any of these factors. These findings suggest complex signaling mechanisms taking place during plant-pathogen interactions.

A set of genes called *hrp* ("harp") (27) controls the ability of phytopathogenic bacteria to cause disease on susceptible plants and to elicit the hypersensitive response on resistant plants (46). Previous studies established the pathological significance of these genes in *Pseudomonas syringae* pv. phaseolicola, the casual agent of halo blight disease in bean plants (27). Most of the *hrp* genes in this bacterium lie in a ~22-kb region (the *hrp* cluster), which is chromosomally located and genetically composed of seven complementation groups, *hrpL*, *hrpAB*, *hrpC*, *hrpD*, *hrpE*, *hrpF*, and *hrpRS* (39) (see Fig. 1). An additional *hrp* gene, *hrpM*, is unlinked to this cluster (12). Several investigators have described genes with similar or analogous functions in phytopathogenic gram-negative bacteria that represent four major taxa: *P. syringae*, *Pseudomonas solanacearum*, *Erwinia* spp., and *Xanthomonas campestris* (6, 7, 26, 35, 44) (for a review, see reference 46).

Little is known about how *hrp* genes function at the biochemical level during pathogenesis or in the elicitation of hypersensitive necrosis. However, progress has been made in understanding *hrp* gene regulation. The *hrpS* gene of *P. syringae* pv. phaseolicola regulates the expression of *hrpD* (16) and other genes in the *hrp* cluster (31). *hrpS* encodes a 34.5-kDa protein which, over most of its length, shares sequence similarity with the central domain of the NtrC subfamily of bacterial regulatory proteins (1). In addition, the *ntrA* gene of *P. syringae* pv. phaseolicola, encoding the σ^{54} factor, is required for the expression of several *hrp* loci (11).

The *Agrobacterium*- and *Rhizobium*-plant interactions provide examples of the apparently complex mechanisms underlying plant-bacterium signal exchange at the infection site (34, 41, 47). In both interactions, specific plant phenolic compounds act as inducers of bacterial genes involved in crown gall pathogenesis and nodulation, respectively (34, 41, 47). In addition, environmental signals such as pH (42, 47) and certain sugars present in the plant (2) act as inducers of *vir* gene expression in *Agrobacterium* species. Although both *Agrobacterium*- and *Rhizobium*-plant interactions are well studied, our knowledge regarding signal transduction in other plant-pathogen interactions is limited.

In this study, we investigated the nature and role of physiological factors involved in the transcriptional regulation of the *hrp* genes of *P. syringae* pv. phaseolicola by using chromosomally located *hrp::inaZ* fusions in complemented and noncomplemented strains grown in planta and in vitro. Our studies show that expression of the *hrp* genes is controlled by a specific plant signal(s), pH, osmolarity, and the nature of the carbon source. The biological significance and the mechanistic implications of these diverse physicochemical inputs in *hrp* gene expression are critically discussed, and a hypothesis concerning the sequence of events which take place early in the plant-bacterium interaction is proposed.

MATERIALS AND METHODS

Media, reagents, and growth conditions. *P. syringae* pv. phaseolicola NPS3121 and all its derivatives were routinely grown at 24°C in King's B medium (21) or in modified King's B medium lacking glycerol as specified in the text and legends. Defined mineral salts media were made by adding various carbohydrates, amino acids, and organic acids from concentrated, filter-sterilized stocks to M9-salts medium (28). Except as otherwise stated in the text and legends, (i) the carbon source used in M9 medium was sucrose and (ii) the final concentrations of carbon sources or other individual supplements were 5 mM and the pH was adjusted to 5.5. Final concentrations of antibiotics in the media were 15, 20, 50, and 100 μg/ml for tetracycline, spectinomycin, nalidixic acid, and rifamycin, respectively.

Transfer from nutritionally complex to defined M9 medium caused a variable growth lag in the cultures depending on the carbon source present in the M9 medium. To minimize this lag, the carbon substrate present in the M9 medium

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant properties[a]	Source or reference
E. coli		
HB101	F⁻ *hsdS20* (*hsdR hsdM*) *recA13 thi leu proA2 lacY1 galk2 rpsL xyl-5 mtl-1 supE44* λ⁻	28
DH5α	F⁻ *endA1 hsdR17*(r_K^- m_K^+) *supE44 thi recA1 gyrA* φ80*lacZ*ΔM15 *relA1* Δ(*lac-proAB-argF*) λ⁻	Bethesda Research Labs
SF800	Nal^r *polA* (Ts) *thy*	W. Paranchych
P. syringae pv. phaseolicola		
NPS3121	Wild type, Rif^r	39
LRE613	NPS3121 *hrpL613*::Tn*3*-spice	39
LR72	NPS3121 *hrpAB72*::Tn*3*-spice	39
LR38	NPS3121 *hrpC38*::Tn*3*-spice	39
LR12	NPS3121 *hrpD12*::Tn*3*-spice	39, 39a
LRA11	NPS3121 *hrpE11*::Tn*3*-spice	39, 39a
LRD71	NPS3121 *hrpF71*::Tn*3*-spice	39
LRG94	NPS3121 *hrpRS94*::Tn*3*-spice	39
RF55	NPS3121 *hrpM55*::Tn*3*-spice; carries an *inaZ* fusion within and in the same direction as the second open reading frame of *hrpM* direction	12
Plasmids		
pPL6	*hrp* cluster clone from the pLAFR3 cosmid library PSP-Lib1 of NPS3121; complements mutations in all genes in the *hrp* cluster	26, 39, and unpublished data
pNN56	*hrp* cluster clone from the pLAFR3 cosmid library PSP-Lib2 of NPS3121; complements mutations in all genes in the *hrp* cluster except *hrpL* and *hrpAB*	39
pC61	*hrpM* region cosmid from PSP-Lib2 and complements only *hrpM* mutants	12
pICE1	pLAFR3 cosmid clone carrying the *iceC* gene from *P. syringae* Cit7	37
pPL10	Carries the 11-kb *Bgl*II fragment inserted in the *Bam*HI site of pLAFR3; complements *hrpE* and *hrpF*	26, 39
pPL11	Carries the 20-kb *Bam*HI-*Hin*dIII fragment from pPL6 cloned in pWB5a; complements *hrpC*, *hrpD*, *hrpE*, and *hrpF*	25, 27, 39
pLR32	Carries the 3.6-kb *Bgl*II fragment spanning *hrpC* and the adjacent portions of *hrpAB* and *hrpD* cloned in pLAFR6; complements only *hrpC* mutants	39
pMMKK1	Carries the ca. 5.2-kb *Kpn*I fragment from cosmid pNN56 inserted in the synonymous sites of the pLAFR6; carries a functional *hrpRS* operon and part of *hrpF*	This study
pMMSR	Carries a 3-kb *Bgl*II-*Bam*HI fragment of the pMMKK1 plasmid inserted in the *Bam*HI site of the pLAFR6; complements *hrpRS* mutants	This study
pMM6AS2	Carries a 6.9-kb *Eco*RV-*Sac*I fragment and complements mutations in *hrpAB* and *hrpC*	39
pLRBR	Carries a 9.3-*Bam*HI-*Eco*RI fragment and complements *hrpC* and *hrpD*	39
pLAFR3	Broad-host-range vector, *incP-1* rlx_{RK2}^+ rep_{RK2}^+ *lacZα cos*⁺ Tet^r	43
pLAFR6	Same properties as those of pLAFR3 but without *lacZα*; contains the multilinker of pUC18 flanked by synthetic *trp* terminators; Tet^r	19
pRK2013	$rep_{El_r}^+$ Δrep_{RK2} tra_{RK2}^+ rlx_{RK2}^+ Kan^r	9
pGEM-7Zf(+)	Amp^r	Promega Corp., Madison, Wis.

[a] Abbreviations: Nal, nalidixic acid; Rif, rifamycin; Tet, tetracycline; Spc, spectinomycin; Str, streptomycin; Amp, ampicillin; Kan, kanamycin.

was also provided to the bacteria during growth in the modified King's B medium to enable the cells to preadapt to that substrate.

Plasmids and strains. Table 1 lists the plasmids described previously and those constructed in this study (see below). The expression of *hrp* genes in King's B and M9 media and in *Phaseolus vulgaris* cv. Red Kidney bean leaves was studied by using the following marker exchange *hrp*::*inaZ* mutants: LRE613 (*hrpL*), LR72 (*hrpAB*), LR38 (*hrpC*), LR12 (*hrpD*), LRA11 (*hrpE*), LRD71 (*hrpF*), and LRG94 (*hrpRS*) (Table 1). These mutants have been described previously (39) and carry single chromosomal insertions of the Tn*3*::spice transposon such that the ice nucleation gene *inaZ* is transcriptionally fused in the direction of *hrp* gene transcription. Additional strains carrying similar fusions in *hrpC*, *hrpF*, and *hrpRS* (one each) and in *hrpAB* and *hrpD* (two each) (39) were included in all experiments and gave similar response patterns to those of the strains listed above (38).

Plasmid constructions. The inserts of relevant plasmids are shown in Fig. 1. pPL6 and pNN56 are cosmids obtained from two different genomic libraries of strain NPS3121, and each spans most of the 22-kb *hrp* cluster (27, 39). To construct plasmid pMMSR, the 5.2-kb *Kpn*I fragment from cosmid PNN56 was first cloned into pGEM-7Zf(+). The resulting plasmid, pMMKK1, was digested with *Bgl*II and *Bam*HI which cleave at the end of the *hrpS* open reading frame (16) and within the polylinker of the vector pGEM-7Zf(+) upstream of *hrpR*, respectively. The 5.2-kb *Bgl*II-*Bam*HI fragment was cloned in the *Bam*HI site of pLAFR6 to produce pMMSR. The pLR32 insert is the 3.6-kb *Bgl*II fragment comprising *hrpC* and the adjacent portions of *hrpAB* and *hrpD* cloned in the *Bam*HI site of pLAFR6 (39).

Plant material. Bean seed (*P. vulgaris* cv. Red Kidney) was obtained from The University of California at Davis. The tobacco plants (*Nicotiana tabacum* cultivar Turkish, 2 to 3 months old) were obtained from The University of

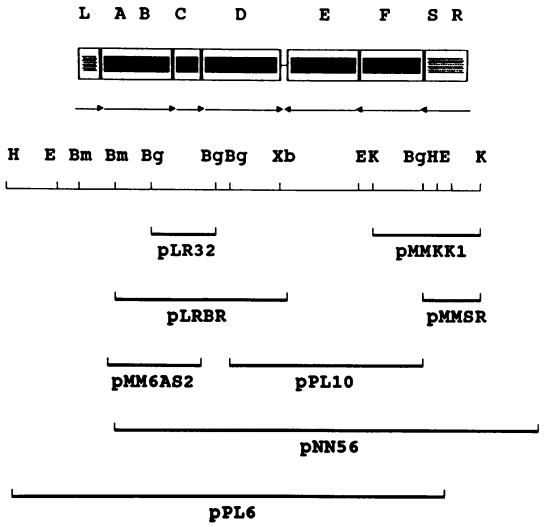

FIG. 1. Genetic and transcriptional organization of the *hrp* gene cluster in *P. syringae* pv. phaseolicola NPS3121 (39) (top). The lines below the restriction map show the inserts of plasmids mentioned in the text. Restriction endonucleases sites: H, *Hin*dIII; E, *Eco*RI; Bm, *Bam*HI; Bg, *Bgl*II; Xb, *Xba*I; K, *Kpn*I.

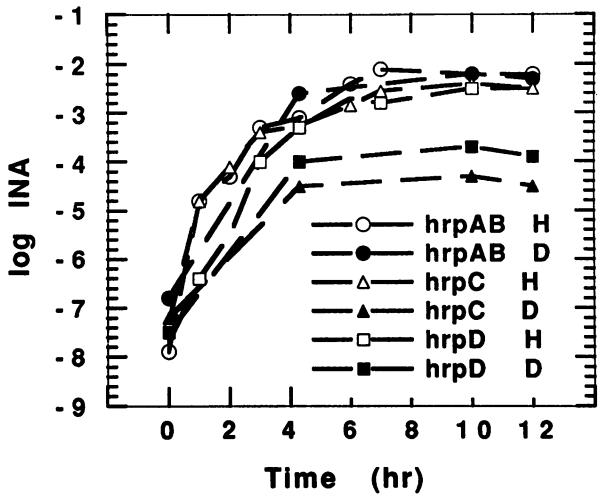

FIG. 2. Time course of induction of chromosomal *inaZ* fusions in *hrpAB* (LR72; circles), *hrpC* (LR38; triangles), and *hrpD* (LR12; rectangles), respectively. At time zero the cultures were transferred from KB-sucrose (5 mM) to M9-sucrose (5 mM, pH 5.5) medium. Zero time values are those recorded in KB medium. Levels of expression are shown for both haploids (H) and merodiploids (M) complemented for the mutant gene function. The complementing plasmids were pMM6AS2, pLR32, and pLRBR for the *hrpAB*, *hrpC*, and *hrpD* mutants, respectively. Open and filled symbols correspond to noncomplemented and complemented strains, respectively. INA is given as the $\log_{10}$ of ice nuclei per CFU. The standard error of the data was 0.3 to 0.5 log INA units.

California at Berkeley. Primary leaves of 8- to 10-day-old bean plants were used for in planta expression studies.

Gene expression assays. The level of expression of *hrp*::*inaZ* fusions in bacterial cultures and plant leaves was quantified by droplet freezing assays as described previously (25, 39). The starter inoculum for both in planta and in vitro experiments consisted of bacterial cultures that had been grown overnight in King's B medium at 24°C, except as otherwise stated. For the in vitro expression experiments, the cell density in the new medium was adjusted to 0.05 to 0.1 optical density unit at 600 nm (Spectronic 20 spectrophotometer, Bausch & Lomb) and the cultures were placed in a shaker incubator at 24°C. Turbidities were measured at the different times the aliquots were removed, and the aliquots were assayed for ice nucleation activity (INA) at −9°C after serial dilution. For in planta expression studies, approximately 10^4 CFU/cm^2 was administered to bean leaves by vacuum infiltration. Tobacco leaves were inoculated as described previously (39) and received 10^7 CFU/cm^2 of leaf. After inoculation, plants were kept in a growth chamber at 24°C under 12-h-light and 12-h-dark periods at 80 and 70% relative humidity, respectively. Samples consisted of two leaf disks (6-mm diameter, 0.28-cm^2 surface area) from each plant. The in vitro and in planta assays were carried out in duplicate, and each experiment was repeated at least thrice.

INA calculation. The INA of strains carrying *hrp*::*inaZ* fusions is expressed in INA units (the number of ice nuclei per CFU) after conversion to logarithm. Previous studies (25, 40) established that, in bacteria such as *E. coli* and *P. syringae* pv. phaseolicola, INA increases as the square power of InaZ monomer concentration up to about 0.4 nuclei per cell (−0.6 log INA units). Above this value, the activity continues to increase with InaZ monomer concentration, but in a linear fashion, up to 1 nucleus per cell (0 log INA units), which is the theoretical upper limit when the assay is carried out in nondisrupted cells (25, 40). Thus, when the activities do not exceed −0.6 log INA units, a 100-fold increase (i.e., a difference of 2 log INA units) corresponds to 10-fold actual induction. Accordingly, the degree of induction was calculated as follows: fold induction = $(\Delta \text{ InaZ activity})^{1/2}$. However, when activities exceed −0.6 log INA units, this calculation underestimates the actual level of expression. The values reported in the figures and tables are averages from three to five different experiments, each involving duplicate samples. Expression levels are given as log INA units plus or minus standard error.

RESULTS

Expression of *hrp* genes in complex and defined media. The INA expressed by haploid strains carrying chromosomal *hrp*::*inaZ* transcriptional fusions in *hrpL* (LRE613), *hrpAB* (LR72), *hrpC* (LR38), *hrpD* (LR12), *hrpE* (LRA11), *hrpF* (LRD71), and *hrpRS* (LRG94) during growth in King's B medium ranged from ≈−7.9 to −5.5 log INA units depending on the operon (Table 2). These levels either did not change significantly or increased by a maximum of about 2.4 log INA units (*hrpL*) when the mutations were complemented.

Subsequently we investigated the expression of *hrp* genes in minimal medium (M9), which more closely simulates the environment of the leaf apoplast and allows different physiological and nutritional parameters to be individually tested. Figure 2 shows the kinetics of induction of *hrp*::*inaZ* fusions in the *hrpAB*, *hrpC*, and *hrpD* loci in both complemented and noncomplemented strains, after transfer from KB-sucrose to M9-sucrose medium adjusted to the optimum pH 5.5 (see below). The expression of these genes was significantly higher in M9 medium, with activities approaching plateau values at 4 to 8 h after transfer from King's B medium. The expression levels of *hrpAB*, *hrpC*, *hrpD*, *hrpE*, and *hrpF* were from 71- to 707-fold higher in M9 medium than in King's B medium in the noncomplemented marker exchange

TABLE 2. Expression levels of *hrp* genes in planta and in vitro[a]

Locus	In planta		In vitro		Fold difference[b]		
	RK	T	M9	KB	RK/M9	T/M9	M9/KB
hrpL							
H	−1.9 ± 0.2	−2.2 ± 0.2	−7.9 ± 0.3	−7.9 ± 0.3	1,000	708	1
D	−1.6 ± 0.2	−3.3 ± 0.3	−4.4 ± 0.2	−5.5 ± 0.4	25	4	4
hrpAB							
H	−0.4 ± 0.2	NA	−2.2 ± 0.3	−7.9 ± 0.3	8	NA	707
D	−0.1 ± 0.3	NA	−2.2 ± 0.2	−6.8 ± 0.3	11	NA	200
hrpC							
H	−0.4 ± 0.2	−1.9 ± 0.3	−2.4 ± 0.3	−7.3 ± 0.2	10	2	282
D	−0.8 ± 0.2	−2.6 ± 0.2	−4.3 ± 0.3	−6.9 ± 0.1	73	7	20
hrpD							
H	−1.5 ± 0.3	−2.0 ± 0.2	−2.5 ± 0.3	−7.7 ± 0.3	3	2	398
D	−0.3 ± 0.3	−2.3 ± 0.2	−3.7 ± 0.3	−7.5 ± 0.3	50	5	79
hrpE							
H	−0.1 ± 0.4	NA	−1.5 ± 0.3	−5.2 ± 0.3	5	NA	71
D	−0.8 ± 0.4	NA	−2.5 ± 0.2	−5.3 ± 0.3	7	NA	25
hrpF							
H	0.9 ± 0.4	NA	−1.0 ± 0.3	−5.5 ± 0.2	>10	NA	158
D	0.1 ± 0.3	NA	−2.4 ± 0.2	−4.0 ± 0.3	18	NA	6
hrpSR							
H	−1.2 ± 0.3	−3.7 ± 0.2	−7.9 ± 0.2	−7.9 ± 0.3	2,239	125	1
D	−2.4 ± 0.2	−3.2 ± 0.2	−6.7 ± 0.2	−7.9 ± 0.3	141	56	4

[a] *inaZ* expression was measured in noncomplemented (haploid [H]) and complemented (merodiploid [D]) strains at 10 h postinoculation in planta and in vitro; activities are expressed as log INA units. The strains used were LRE613 (*hrpL*), LR72 (*hrpAB*), LR38 (*hrpC*), LR12 (*hrpD*), LRA11 (*hrpE*), LRD71 (*hrpF*), and LRG94 (*hrpRS*). The complementing plasmids were pPL6 for LRE613, pMM6AS2 for LR72, pLR32 for LR38, pPL10 for LRA11 and LRD71, and pMMSR for LRG94. Abbreviations: RK, cultivar Red Kidney bean leaves; T, cultivar Turkish tobacco leaves; M9, M9 salts plus 5 mM sucrose, pH 5.5; KB, King's B medium.

[b] Calculated as described in Materials and Methods.

haploids and from 6- to 200-fold higher in the complemented merodiploids, depending on the gene. In general, lower levels of expression were observed with the complemented strains than with the noncomplemented strains, except for *hrpAB* where the expression levels were essentially equal regardless of the functional state of the gene (Table 2 and Fig. 2). One reason for this decrease may be activator titration caused by the additional *hrp* gene promoters present in the complementing plasmids. In these experiments, the merodiploids carried plasmids able to complement the mutation of interest while lacking active copies of *hrpRS* and *hrpL*, which are regulatory genes (31) and might alleviate the effects of activator titration. The above results show that *hrpAB*, *hrpC*, *hrpD*, *hrpE*, and *hrpF* are actively transcribed in M9 medium, and the products of these genes are not required for their own induction.

In M9 medium, the behavior of *hrpL* and *hrpRS* differed in comparison to that of the other five genes. The haploid strains LRE613 (*hrpL*::*inaZ*) and LRG94 (*hrpRS*::*inaZ*) gave *inaZ* activity levels in the lowest detection range, both in King's B and M9-sucrose media (Table 2). Expression of *hrpL*, but not *hrpRS*, was detected in King's B medium only when the mutation was complemented. The complemented *hrpL* and *hrpRS* strains showed low but reproducible expression in M9 medium (roughly 4.0-fold higher than that in King's B medium) (Table 2 and Fig. 3A and B). Thus, *hrpL* and *hrpRS* are transcribed in M9 medium but at much lower levels than those of the other five *hrp* genes. Furthermore, the *hrpRS* operon requires its functional product(s) in order to be expressed in vitro. Whether or not this is true for *hrpL* cannot be concluded with certainty since the complementing plasmid carried an active *hrpS* gene.

Plant induction. As reported previously, strains containing a functional *hrp* region show considerably higher levels of *hrp*::*inaZ* fusion expression in bean leaves relative to the levels obtained in King's B medium (39). To extend this study, we investigated the expression of the seven *hrp* operons by using *hrp*::*inaZ* fusions in bean leaves and compared the expression levels with those obtained in M9 medium, with both complemented and noncomplemented strains. In addition, we examined the expression of *hrpL*, *hrpC*, *hrpD*, and *hrpRS* gene fusions in tobacco leaves to see whether the *hrp* genes are regulated similarly or differently in the compatible and incompatible interactions.

Figures 3A and B show the course of induction of *hrpL* and *hrpRS* in bean leaves and M9 medium. The INA levels in bean leaves relative to those in M9 medium were 1,000- and 2,239-fold higher in the noncomplemented *hrpL* and *hrpRS* mutants, respectively (Table 2). Thus, *hrpL* and *hrpRS* were expressed in bean leaves independently of their functional state, suggesting that different mechanisms are involved in the regulation of these operons in planta versus those in vitro. The other five operons of the *hrp* cluster also gave higher maximum levels of expression in planta compared with the maximum levels determined in M9 medium (Fig. 3C and Table 2). In general, the noncomplemented mutants showed from 3- to ≥10-fold greater induction and the complemented strains showed from 7- to 73-fold greater induction in planta than they did in M9 medium (Table 2). The actual induction levels of *hrpAB*, *hrpC*, *hrpD*, *hrpE*, and *hrpF* in bean leaves may be higher than those calculated because the maximum INA activity levels fell in the linear range of the *inaZ* reporter system (above −0.6 log or 0.4 INA units) or approached its theoretical maximum (0 log or 1 INA units). In contrast, the induction levels of *hrpL* and *hrpRS* could be more accurately determined because their maximum levels remained well below the theoretical maximum and within the quantifiable range of the *inaZ* system.

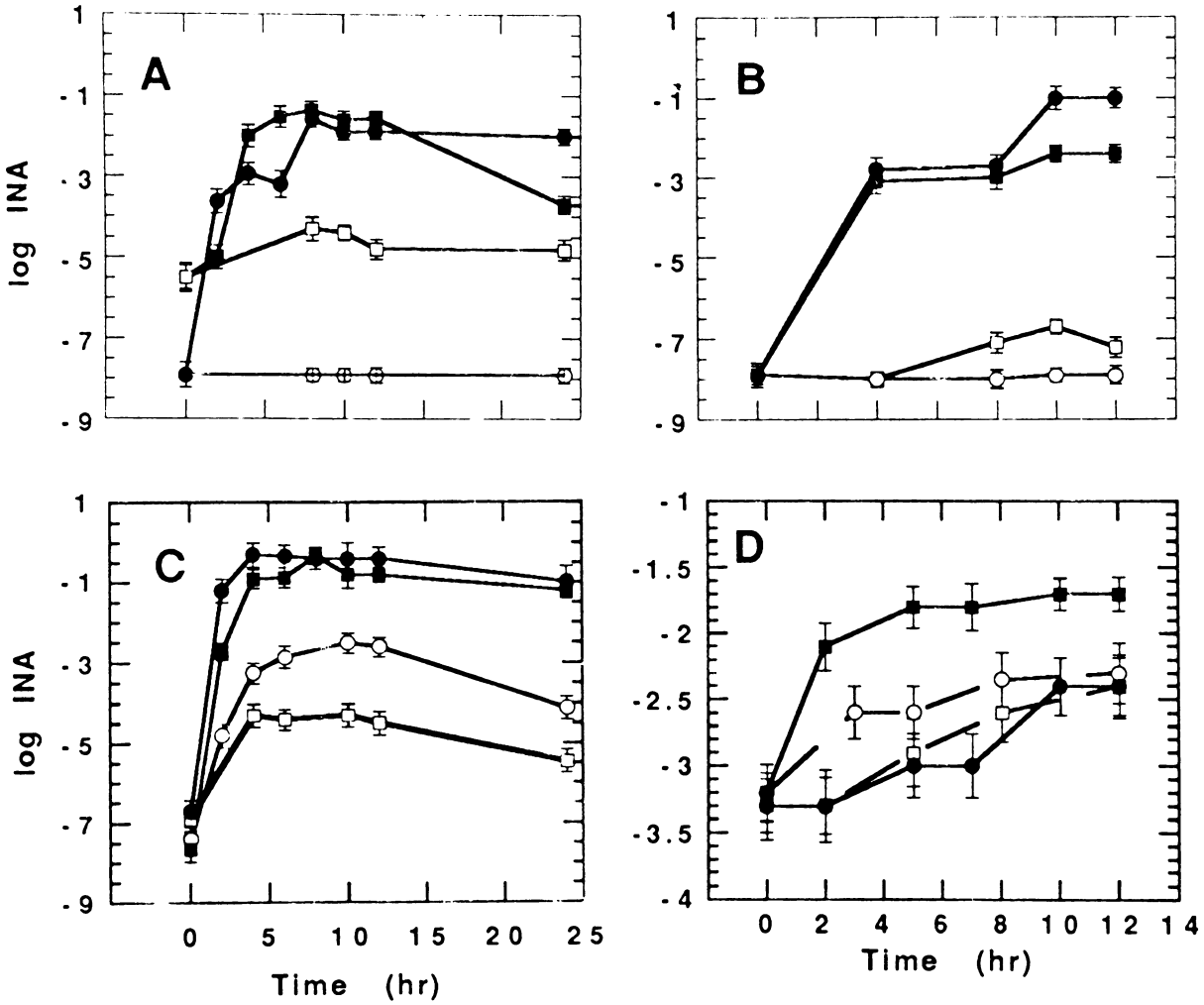

FIG. 3. Time course of expression of *hrpL*::*inaZ* (LRE613) (A), *hrpRS*::*inaZ* (LRG94) (B), and *hrpC*::*inaZ* (LR38) (C). Levels of expression recorded in M9-sucrose medium (open symbols) and in cultivar Red Kidney bean leaves (filled symbols) are shown for both haploids (circles) and merodiploids (rectangles) complemented for the mutant gene function. The complementing plasmids were pPL6, pMMSR, and pLR32 for *hrpL*, *hrpS*, and *hrpC*, respectively. (D) Time course of *iceC* and *inaZ* expression in strains NPS3121 (pICE1) (rectangles) and RF55 (pC61) (*hrpM*::*inaZ*; circles) in M9-sucrose medium (open symbols) and in cultivar Red Kidney bean leaves (filled symbols).

The *hrpL*, *hrpC*, *hrpD*, and *hrpRS* gene fusions were also actively induced in tobacco leaves. However, the levels of induction were slightly lower in tobacco leaves than in bean leaves. Overall, the induction levels at 10 h postinoculation (just before the onset of hypersensitive collapse seen with the complemented strains) exceeded those observed in M9 medium by 2- to 708-fold for the noncomplemented strains and 4- to 56-fold for the complemented strains (Table 2). Therefore, all of the gene fusions tested were expressed at higher levels in bean and tobacco leaves than they were in M9 or King's B medium regardless of whether the strains bearing these fusions contained a complemented *hrp* region.

Strain NPS3121(pICE1), expressing the *iceC* gene from its own promoter, and RF55(pC61), carrying a *hrpM*::*inaZ* fusion in the chromosome, were included in these experiments for comparison. These strains showed similar levels of expression in King's B (data not shown) and M9 media and only slight induction in bean leaves (Fig. 3D). These results show that the induction of the seven operons in the *hrp* cluster represents an actual increase in the level of transcription and is not a result of effects exerted on the ice nucleation protein itself or on ice nucleus function. Therefore, the above experiments provide clear evidence that a plant signal(s) is specifically involved in the activation of the *hrp* genes in the 22-kb cluster.

Analysis of factors affecting the *hrp* gene expression in vitro. It is well established that the physiological conditions in the plant apoplast (e.g., pH, ion, and organic solute composition) change dramatically during the course of compatible and incompatible interactions (3, 8, 14). The scope of our experiments described below was to study the effects of some of these factors individually on the expression of the five *hrp* operons that showed significant expression under in vitro conditions.

(i) Regulation of *hrp* gene expression by pH. Figure 4 illustrates the effect of pH on the expression of *hrpAB*, *hrpC*, *hrpD*, *hrpE*, and *hrpF* after transfer from King's B medium (pH 7.5) to M9 medium adjusted to pH 5.5, 6.5, 7.5, and 8.5 (pH values lower than 5.5 or higher than 8.5 did not support normal bacterial growth). The maximum expression of all

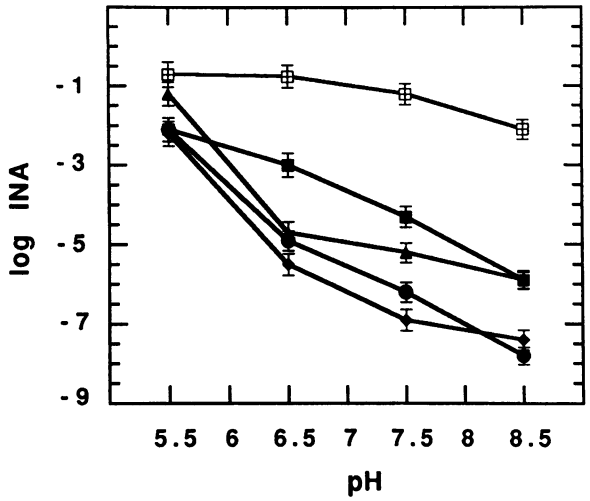

FIG. 4. Effect of pH in the range 5.5 to 8.5 on the expression of *hrpAB* (LR72; circles), *hrpC* (LR38; filled rectangles), *hrpD* (LR12; diamonds), *hrpE* (LRA11; triangles), and *hrpF* (LRD71; open rectangles) in M9 medium containing 5 mM sucrose at 10 h after transfer from KB medium.

hrp genes was obtained at pH 5.5. The expression of *hrpAB*, *hrpC*, *hrpD*, and *hrpE* was strongly reduced as the pH was increased above pH 6.5. In contrast, the expression of *hrpF* was not influenced significantly by pH (Fig. 4). When the pH of the King's B medium was adjusted to 5.5, the expression of the genes described above increased by a maximum of 2 log INA units relative to their expression at pH 7.5 (data not shown). This suggests that other factors besides pH are involved in *hrp* gene regulation.

(ii) Osmoregulation of the *hrp* genes. The effect of osmotic strength on the expression of *hrpAB*, *hrpC*, *hrpD*, *hrpE*, and *hrpF* was tested by adding increasing concentrations of NaCl to M9 medium adjusted to pH 5.5. Increasing concentrations of NaCl progressively reduced the expression of *hrpAB*, *hrpC*, and *hrpD*, and 150 mM NaCl caused complete repression of these genes (Fig. 5A). In contrast, the expression of *hrpE* was slightly reduced, while the expression of *hrpF* was not affected. Concentrations of NaCl up to 200 mM did not adversely affect the growth of either the wild-type strain NPS3121 or any of the *hrp* mutants. Therefore, the lower levels of INAs observed reflect an actual decrease in the expression of the *hrp* genes rather than an inhibition of growth.

To determine whether the repression observed with NaCl was a general reflection of osmotic strength rather than being NaCl specific, several other osmolytes were similarly tested. Figure 6 compares the effects of five different substances ($MgCl_2$, $MgSO_4$, KCl, sorbitol, and sucrose) on *hrpAB* expression, on the basis of their relative osmosities (45). All five substances caused repression but to a different degree at a given osmosity value. These differences may be related to the physiological state of the cells, since each osmolyte challenges the uptake systems, ionic balance, and adaptive responses of the cells in different ways. Overall, there was a strong positive correlation between osmosity and the degree of repression. Similar results were obtained with *hrpC* and *hrpD* (38).

The fact that *hrpF* was not osmotically repressed shows that colligative effects due to high solute concentrations did not interfere with the freezing assays and high concentrations of osmolytes did not have any effect on ice nucleus assembly and function. This is further borne out by the results from similar experiments with two different ice nucleation genes, each expressed from a different promoter. Strain NPS3121(pICE1) carries the *iceC* gene driven by its own promoter, and strain RF55 contains a chromosomal *inaZ* insertion in the *hrpM* gene. Both strains expressed similar levels of ice nucleation activity when grown in the presence or absence of 150 mM NaCl (Fig. 5B).

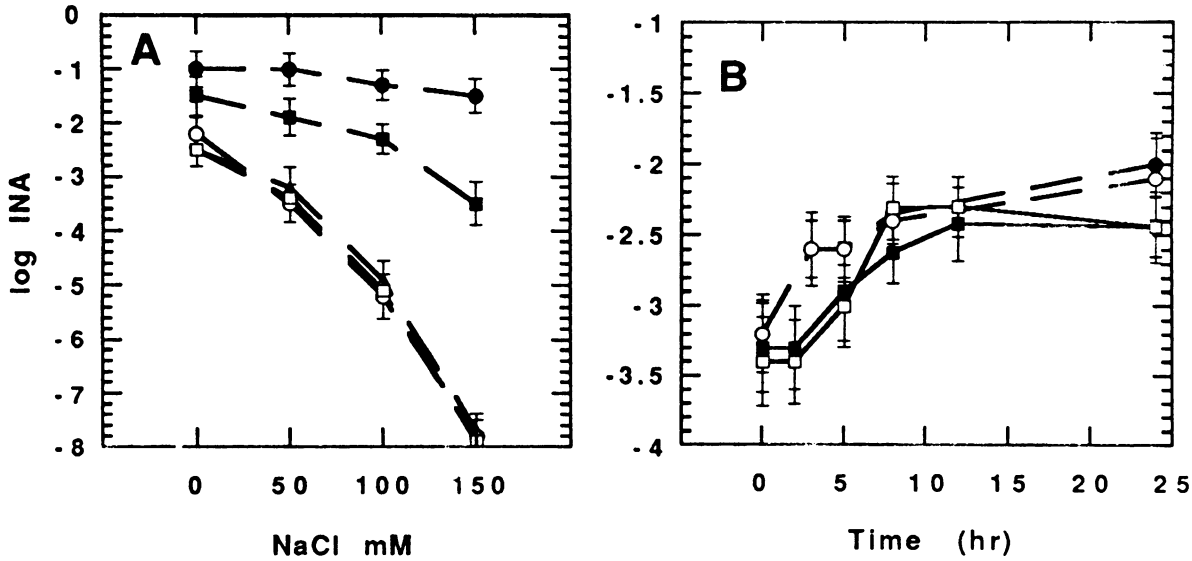

FIG. 5. (A) Expression levels of *hrpAB* (open circles), *hrpC* (open rectangles), *hrpD* (triangles), *hrpE* (filled rectangles), and *hrpF* (filled circles) at 10 h after transfer from KB medium to M9 medium containing 5 mM sucrose and 0, 50, 100, and 150 mM NaCl. (B) Time course of *iceC* and *inaZ* expression in strains NPS3121 and RF55 (*hrpM*::*inaZ*) carrying plasmid pICE1 (circles) and the functionally complementing plasmid pC61 (rectangles), respectively. The medium was M9-sucrose with and without 150 mM NaCl (open and filled rectangles, respectively).

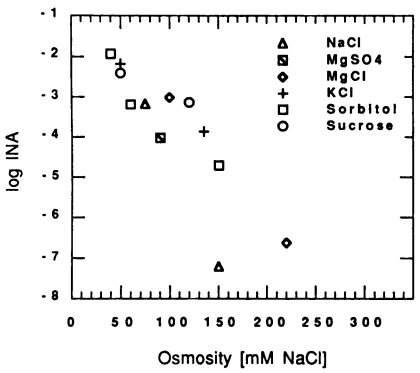

FIG. 6. Effectiveness of different osmolytes in causing repression of *hrpAB*::*inaZ* in strain LR72. Levels of expression (in log INA units) at 10 h after transfer from KB medium to M9 medium containing different osmolytes are plotted against osmosity (the molar concentration of NaCl with the same osmotic pressure as a given solution of a different solute [45]). The standard error of the data was 0.3 to 0.6 log INA units.

(iii) Carbohydrates, organic acids, and amino acids. The levels of expression of *hrpAB*, *hrpC*, *hrpD*, *hrpE*, and *hrpF* 12 h after transfer from King's B to M9 medium containing organic acids (succinate or citrate) or carbohydrates (fructose or sucrose) at 5 mM concentrations as the sole carbon sources are shown in Fig. 7. In succinate-containing medium, the expression levels of all five genes were lower than the levels obtained in medium containing citrate or either sugar. Citrate-grown cells also showed lower expression of *hrpC*. When citrate or succinate was added to fructose- or sucrose-containing media, the expression of *hrpAB*, *hrpC*, and *hrpD* was reduced by 2 to 2.5 log INA units and similar effects were observed with pyruvate or α-ketoglutarate on *hrpD* expression (38). Thus, organic acids decreased the expression of *hrpAB*, *hrpC*, *hrpD*, and *hrpE* but affected *hrpF* to a lesser extent. Tricarboxylic acid cycle intermediates are the preferred substrates in comparison to carbohydrates in *P. syringae* pv. phaseolicola (31) and in other *Pseudomonas* species (24), suggesting that the underlying mechanism is probably catabolite repression. We also tested the effect of various protein amino acids as the sole sources of carbon in M9 salts medium or as supplements to sucrose on the expression of *hrpAB*, *hrpC*, and *hrpD* and found levels of expression comparable to those in M9-sucrose medium (generally, the differences varied from undetectable to ≈1.3 log INA unit) (38). An exception was *hrpC*, whose expression was 2 to 2.5 log INA units lower on the glutamate medium than on the sucrose medium.

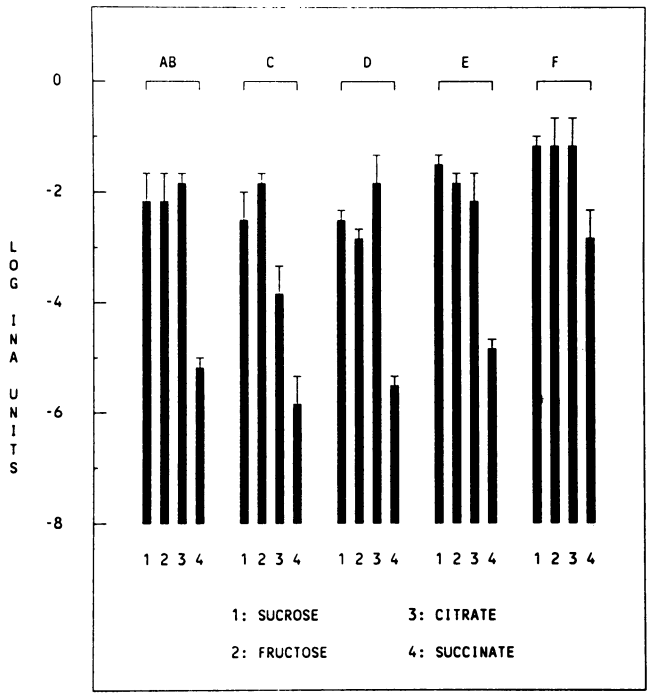

FIG. 7. Levels of *inaZ* expression of *hrpAB* (LR72), *hrpC* (LR38), *hrpD* (LR12), *hrpE* (LRA11), and *hrpF* (LRD71) at 10 h after transfer from King's B to M9 medium containing different carbohydrates or organic acids as the sole sources of carbon at a concentration of 5 mM. The cells were grown overnight in King's B medium and preadapted to the carbon source used in M9 medium as described in Materials and Methods.

DISCUSSION

The pathological significance of *hrp* genes in four major taxa of plant pathogenic prokaryotes (*P. syringae*, *P. solanacearum*, *Erwinia* spp., and *X. campestris*) is now well established (46). In a previous study (39), we reported that all genes or operons in the *hrp* cluster of *P. syringae* pv. phaseolicola are induced in bean leaves. The present investigation was undertaken to evaluate the effects of physiological and environmental factors on the expression of *hrp* genes and to determine whether substances originating in the plant enhance their transcriptional activity.

Our studies showed that *hrp* gene expression is under the control of a plant signal(s) and of multiple physiological and environmental factors, specifically, pH, osmotic strength, and catabolite repression. The optimum expression of all *hrp* genes in vitro occurred under conditions that simulate the environment of the leaf apoplast with respect to low osmolyte content and pH 5.5 (13, 15, 30, 36). Under the optimal conditions, the expression levels in vitro were substantially lower than those in both bean and tobacco plants. The differences were most pronounced with *hrpL* and *hrpRS* marker exchange haploids, which expressed no detectable activity under any in vitro conditions that we tested but gave very high levels of activity in bean and tobacco leaves. We hypothesize that either a chemical substance(s) or another signal(s) originating in the plant is responsible for the elevated levels of *hrp* gene expression in planta. Preliminary results (31) showed that strains carrying *hrpRS*::*inaZ* fusions express significant levels of INA when grown in the presence of tobacco cell suspension cultures as well as in the supernatant from which the tobacco cells have been removed but no detectable activity in the cell culture medium alone. These observations support the above hypothesis and further suggest that a plant signal molecule(s) is an important component of the signal transduction pathway that leads to the high levels of *hrp* gene expression observed in plant leaves.

The lower expression levels observed with the functionally complemented strains in tobacco plants relative to those of bean plants (incompatible and compatible interaction, respectively) can be partly attributed to electrolyte leakage and pH increase associated with incompatible interactions (3, 8, 22). This hypothesis does not explain the differences in the expression levels obtained with the marker exchange

haploids, since these strains did not elicit visible phenotypic responses and *hrp* mutants cause neither electrolyte leakage nor pH changes in the leaf mesophyll (4). The differences between bean and tobacco plants could reflect qualitative and/or quantitative variations of the putative plant signal(s) hypothesized above.

Bacterial genes which respond to plant signals and environmental or physiological factors have been previously described for other phytopathogenic and plant symbiotic bacteria (5, 20, 33, 34, 42). The *vir* genes of *Agrobacterium tumefaciens*, the *nod* genes of *Rhizobium* spp., and the *syrB* gene of *P. syringae* pv. syringae are all modulated by plant phenolic compounds (33, 34, 42). In addition, the *vir* genes of *A. tumefaciens* are regulated by environmental factors including pH (42). Monosaccharides also induce the expression of *vir* genes (2) and *syrB* (33). Substrate effects suggestive of catabolite repression have been reported previously in the case of an avirulence gene (*avrB*) in the related pathogen *P. syringae* pv. glycinea (19).

In addition, our results indicate that *hrpL* and the *hrpRS* operon are controlled through distinct regulatory mechanisms in vitro versus those in planta, since in marker exchange haploids these genes were expressed only in leaves. Furthermore, *hrpRS* and possibly *hrpL* are positively autoregulated only in vitro. Previous studies have shown that *hrpS* is positively required for the expression of *hrpD* (16), and data to be published show that *hrpL* and *hrpRS* are also required for the expression of the other genes in the *hrp* cluster (31). Positive autoregulation of regulatory operons has been previously observed with other bacteria that interact with plants, such as *Agrobacterium* and *Rhizobium* spp. (47). However, the regulation of *hrpL* and *hrpRS* may represent a unique case since our data indicate that at least two distinct mechanisms are involved in their regulation.

The osmotic repression and the multiple environmental control of the *hrp* gene expression in *P. syringae* pv. phaseolicola constitute novel observations for this group of genes. Osmotic control of virulence-related genes has been described in human pathogenic bacteria (10, 29). The molecular basis of osmoregulation is complex, and one of the mechanisms proposed to be involved is the alteration of the topological state of DNA (10, 18, 32), which is also brought about by other types of stress (10). The molecular basis of osmoregulation and the low pH response of *hrp* genes merit investigation.

Biological significance of regulatory mechanisms. The complex regulation of the *hrp* genes may be related to the fact that they are indispensable for both compatible and incompatible interactions. In the following paragraph, we consider a plausible scenario regarding the biological significance of these adaptations in the context of the plant-bacterium interactions.

The modulation of *hrp* gene expression by a plant signal(s), osmolarity, pH, and nutrients suggests a complex set of adaptations to potential signal inputs which *P. syringae* pv. phaseolicola experiences at various stages of the infection in the leaf intracellular space. The plant leaf apoplast is a hypo-osmotic environment (15, 36) of low pH (i.e., 5.5) (13, 15) and limited nutrient concentration (17). As shown in this work, these conditions cause partial induction of several *hrp* genes, and this may represent a form of signaling that precedes or is coincident with the early stages of infection, perhaps alerting the pathogen to the presence of a potential host. Concurrent with or subsequent to this induction, active communication between the bacterium and the plant, possibly mediated by a putative plant signal(s) hypothesized above, maximizes *hrp* gene expression to levels that can fully sustain infection. A different set of events can be envisioned in the case of incompatible interaction. During a resistance response, a massive leakage of solutes and a net increase in K^+ efflux/H^+ influx (the XR reaction) from the plant cell to the apoplast take place (3, 8, 22). This increases the osmosity and the pH in the leaf intercellular space and creates repressive conditions for *hrp* gene expression. These repressive conditions may represent a front line defense against the pathogen, until other plant defense mechanisms are fully deployed (23). Analogous arguments may be advanced for the restriction of lesion size at late stages of disease development, where similar events take place (14).

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