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A *Xanthomonas* Pathogenicity Locus Is Induced by Sucrose and Sulfur-Containing Amino Acids

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Expression of *hrp* (*h*ypersensitive *r*eaction and *p*athogenicity) genes from *Xanthomonas campestris* pv *vesicatoria* is suppressed in complex media but induced in the plant. We examined the effects of macronutrients on transcription of *hrp-gusA* (β-glucuronidase) fusions by growth of the bacteria in defined medium. Modified MM1 minimal medium, supplemented with casamino acids, was able to induce *hrpF* strongly when sucrose or fructose was added as a carbon source. However, high concentrations of casamino acids suppressed *hrpF* induction. Sulfur-containing amino acids were required for induction, with methionine induction being comparable to induction in plants. Both sucrose and methionine were required for induction. Induction in medium optimal for *hrpF* induction, designated XVM1, occurred at pH 5.5 to pH 7.5. High concentrations of phosphate or sodium chloride suppressed gene activation. Gene induction was inhibited by succinate, citrate, pyruvate, and glutamine. Expression levels of different *hrp* loci from *X. c. vesicatoria* in XVM1 varied, dependent on the genetic background of the *Xanthomonas* strain used. The results suggest that several control mechanisms might be involved in the expression of *hrp* genes.

INTRODUCTION

Xanthomonas campestris pv *vesicatoria* is the causal agent of bacterial spot disease of pepper and tomato. After infection of a susceptible plant, the bacteria grow in the intercellular space of leaves, giving rise to necrotic lesions. The *hrp* (*h*ypersensitive *r*eaction and *p*athogenicity) genes of *X. c. vesicatoria* are required for both the pathogenic interaction with the host plant and for the induction of the hypersensitive response in resistant host and nonhost plants (Bonas et al., 1991). Genetic analysis and complementation studies showed that the *hrp* genes of *X. c. vesicatoria* are organized into at least six complementation groups. These loci, designated *hrpA* to *hrpF*, are localized in a chromosomal region of ~25 kb. Transposon insertions into any of the *hrp* loci abolish growth of the bacteria in susceptible plants and result in the inability to cause any visible phenotypic reaction in susceptible or resistant plants (Bonas et al., 1991).

The biochemical function(s) of the *hrp* genes from *X. c. vesicatoria* is unknown. *hrp* Genes, first identified in *Pseudomonas syringae* pv *phaseolicola* by Lindgren et al. (1986), and operationally defined by their mutant phenotype, have been isolated from different phytopathogenic bacteria, including subspecies of *Pseudomonas, Xanthomonas,* and *Erwinia* (Willis et al., 1991).

We studied the expression of the *hrp* loci from *X. c. vesicatoria* at the RNA level and by using gene fusions to the β-glucuronidase (*gusA*) gene (Schulte and Bonas, 1992). Growth of the bacteria under different environmental conditions demonstrated that the *hrp* loci are activated during growth in the plant but are repressed in complex and M9 minimal medium. Furthermore, filtrates of pepper, tomato, and tobacco cell suspension cultures contain molecules that induce *hrp* gene expression. These factor(s) were partially purified from tomato-conditioned medium (TCM) and found to be small, organic, heat stable, and hydrophilic. The ability of TCM to induce *hrp* gene expression might be due to the presence of inducing factor(s) and/or the provision of balanced nutritional conditions. After size-fractionation of TCM on biogel P2, the inducing activity was present in fractions containing compounds of low molecular weight (Schulte and Bonas, 1992). Further attempts to purify the fractions resulted in loss of inducing activity or inability to support growth of the bacteria (R. Schulte, unpublished results).

Knowledge of the nature of factors regulating *hrp* gene expression in phytopathogenic bacteria is limited, in part due to the fact that the composition of the nutrients available in the plant is unknown. In a number of different bacteria, *hrp* genes were found to be induced in minimal media without any plant factor. For *P. s. glycinea*, a defined minimal medium induces expression of the avirulence gene *avrB* to levels comparable with those observed in bacteria grown in the plant. The *avrB* gene is under the control of *hrp* genes (Huynh et al., 1989), and the same medium induced *hrp* genes from *P. s. syringae* (Huang et al., 1991). Recently, *hrp* genes were isolated from *X. c. campestris* and shown to be repressed in complex medium but induced in minimal medium (Arlat et al.,

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1991). *hrp* Loci from *P. solanacearum* are induced in minimal medium conditions (Arlat et al., 1990), and *hrp* loci from *P. s. phaseolicola* are induced by M9 medium (Fellay et al., 1991). We have tried, therefore, to establish a defined minimal medium for *hrp* gene induction in *X. c. vesicatoria.*

In this article, we describe a defined, minimal medium that is able to support growth of *X. c. vesicatoria* and to induce expression of the *hrpF* locus to levels comparable to those obtained in the plant. Induction was found to be dependent on (1) low concentrations of both phosphate and sodium chloride, (2) the provision of sucrose or fructose as a carbon source, and (3) the presence of a sulfur-containing amino acid.

RESULTS

Search for a Defined Medium That Does Not Suppress the Induction of *hrpF*

Previously, the *hrp* genes were found to be induced not only by growth of the bacteria in the plant but also by factors in filtrates recovered from TCM (Schulte and Bonas, 1992). We wished to determine whether *hrp* genes could be induced in the absence of plant-derived molecules. Therefore, we attempted to establish a defined medium that would allow efficient induction of *hrp* gene expression and support growth of *X. c. vesicatoria.* An essential feature of the medium was that it should not suppress TCM induction. *X. c. vesicatoria* strain 85-10 (pF312) was chosen as the test strain (plasmid pF312 carries a transcriptional *hrpF-gusA* fusion). In comparison to the gene activity of other *hrp* loci, this strain gave the highest level of β-glucuronidase (GUS) activity after growth in the plant or in TCM, i.e., values in the range of 70 to 100 × 10^{-10} units per colony-forming unit (cfu). No expression of *hrpF-gusA* was detected when the bacteria were grown in either complex medium (NYG), in M9 minimal medium, or in MS (Murashige and Skoog, 1962), the medium used for the generation of TCM (Schulte and Bonas, 1992). Addition of M9 to TCM strongly suppressed the inducing activity. To identify the components that were responsible for this suppression, single components of the minimal media M9 and MM1 (Roy et al., 1988), described for growth of *X. campestris* (de Crecy-Legard et al., 1990), were added to TCM. TCM was used in a 10-fold dilution in water, still giving 75% of its original activity. GUS activity was determined 14 hr after inoculation of the test strain 85-10 (pF312) into the modified TCM. The results are summarized in Table 1. Induction of GUS activity in pure TCM was ~70 × 10^{-10} units per cfu. Addition of thiamine (1 to 10 μg/mL), present at 10 μg/mL in M9, or casamino acids (1.5 g/liter) reduced the inducing activity of TCM to 1 to 5%. Potassium phosphate (10 to 50 mM) also suppressed *hrpF* induction, whereas addition of the other components (see Methods) did not have a negative effect. Suppression of *hrpF* induction by addition of casamino acids or phosphate to TCM was not observed when the concentration of these components was reduced to 0.3 g/liter and 0.5 mM (0.16 mM KH_2PO_4, 0.32 mM K_2HPO_4), respectively. Addition of a modified MM1 (0.5 mM phosphate; 20 mM NaCl) to TCM did not suppress *hrpF* induction.

Table 1. Effect of Added Solutes on Growth and Inducibility of GUS Activity of *X. c. vesicatoria* Strain 85-10 (pF312) in TCM

Medium or Component Added to TCM	Growth	GUS Activity (× 10^{-10} units cfu^{-1})[a]
None	+	70.0
M9 or MM1[b]	+	0.2
24 mM phosphate	+	3.3
0.5 mM phosphate	+	70.0
Thiamine (1–10 μg/mL)	+	2.0
Casamino acids (1.5 g/liter)	+	1.0
Casamino acids (0.3 g/liter)	+	40.0
Modified MM1[c]	+	100.0
Sucrose	+	120.0

[a] Units were calculated as described in Methods. The results are the mean of two independent experiments performed upon two different samples in each case.
[b] Supplemented with sucrose (10 mM).
[c] Modified MM1 medium, with 20 mM NaCl and 0.5 mM phosphate.

Effects of Amino Acids on Induction

MM1 medium contains micronutrients that might be important for growth of *X. c. vesicatoria*. As described above, modified MM1 (0.5 mM phosphate; 20 mM NaCl) did not suppress *hrpF* induction when added to TCM. We therefore used this defined medium as basal medium to test whether *hrpF* could be induced by addition of selected organic compounds. Sucrose (10 mM) was used as sole carbon source. No induction of *hrpF* was detected under these conditions. When casamino acids were added as organic nitrogen source, *hrpF* was strongly induced but only when casamino acids were present in low concentrations (0.1 to 0.3 g/liter). As shown in Figure 1, the highest GUS activity was in the range of 50 to 60 × 10^{-10} units per cfu. In the presence of ≥0.5 g/liter casamino acids, induction of *hrpF* was suppressed. The suppression by casamino acids was also observed in the presence of 50 mM sucrose (Figure 1).

To examine which amino acid(s) might be responsible for *hrpF* induction, single amino acids were added to modified MM1 containing sucrose. Induction of GUS activity was observed only with L-cysteine and L-methionine, producing GUS activities of ~2.9 × 10^{-10} and ~30 × 10^{-10} units per cfu, respectively. For the other amino acids, the values were in the range of background activity (0.5 × 10^{-10} units per cfu or less).

To test whether a sulfur-containing amino acid alone could induce *hrp* and act as a carbon source, methionine was

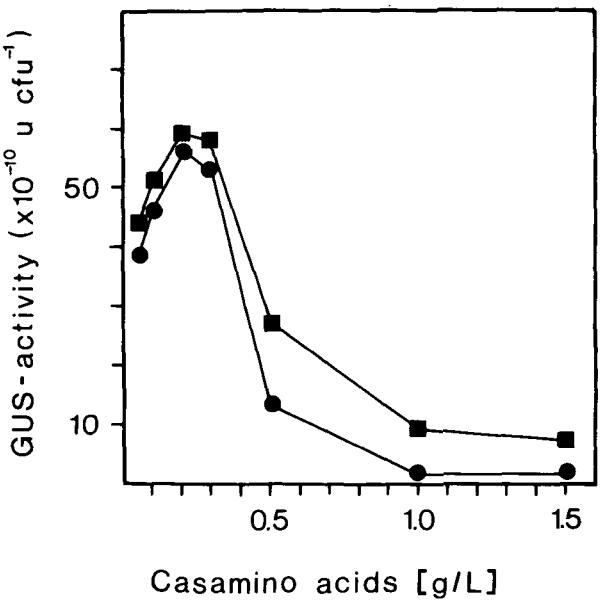

Figure 1. Dependence of *hrpF* Induction on Low Concentration of Casamino Acids.

GUS activity of strain 85-10 (pF312) was determined after growth for 14 hr in modified MM1 (20 mM NaCl; 0.5 mM phosphate), supplemented with different concentrations of casamino acids. Sucrose was present at 10 (●) or 50 (■) mM. Units were calculated as described in Methods. The results are the mean of two independent experiments performed upon two different samples in each case. Uninduced levels are below 0.1 × 10^{-10} units/cfu.

added to modified MM1 without sucrose at final concentrations of 0.0005 to 8 mg/mL. No induction was detected. The inducing effect of the sulfur-containing amino acids was analyzed more closely. In addition to methionine, cysteine, and cystine, the tripeptide glutathione, which contains cysteine, in the reduced and oxidized form was tested in modified MM1 + sucrose. Each of these compounds induced GUS activity. Because induction by methionine was 10-fold higher than by cysteine, cystine, and both forms of glutathione, methionine was used in further experiments.

Induction by Different Carbon Sources

Low molecular weight fractions of TCM contained *hrp*-inducing factor(s). Gas chromatographic and mass spectroscopic analysis of these fractions showed the presence of a number of different carbohydrates. Commercially available carbohydrates were added at 10 mM to modified MM1 containing methionine, and subsequently tested for ability to support growth of strain 85-10 (pF312) and induction of GUS activity. The results are summarized in Table 2. Only fructose, glucose, mannose, and sucrose induced *hrpF*. In addition, they supported bacterial growth. Induction by mannose was 5% of the level for sucrose. Other compounds, such as cellobiose, xylose, and mannitol, enabled the bacteria to grow but did not induce expression of *hrpF*.

To compare induction by sucrose, fructose, and glucose, bacteria were grown in the modified MM1 medium + methionine, supplemented with 1 to 100 mM sugar. As shown in Figure 2, induction of *hrpF* with fructose or sucrose was much better than with glucose, reaching maximum induction levels at ~10 mM.

For subsequent studies of *hrp* gene induction, we used the modified MM1 (20 mM NaCl, 0.5 mM phosphate, 10 mM sucrose, 2 μg/mL methionine), called XVM1, as a basal medium. Strain 85-10 (pF312) was tested for induction by growth of the bacteria on XVM1-agarose plates (see Methods for details). The *hrpF-gusA* fusion showed GUS activity by fluorescence under UV light. Induction of *hrpF* was also observed when a null *hrpF* mutant was tested, i.e., in the absence of a functional *hrpF* locus.

We tested whether different carbon sources suppress *hrpF* induction by sucrose in XVM1. The bacteria were grown in

Table 2. GUS Activity and Growth of *X. c. vesicatoria* 85-10 (pF312) in Modified MM1 (20 mM NaCl and 0.5 mM phosphate), Supplemented with Methionine (2 μg/mL) and Different Carbon Sources[a]

Carbon Source	GUS Inducibility[b]	Growth
D-altrose	–	–
L-(+)-Arabinose	–	–
β-D-(–)-Fructose	+ +	+
D-(+)-Galactose	–	–
L-(–)-Galactose	–	–
D-(–)-Glucose	+	+
D-(–)-Lyxose	–	+
L-(+)-Lyxose	–	–
D-(+)-Mannose	+/–	+
D-(–)-Sorbose	–	–
L-(–)-Sorbose	–	–
D-(+)-Talose	–	–
D-(+)-Xylose	–	+
Methyl-α-D-glucopyranoside	–	–
Methyl-β-D-glucopyranoside	–	–
Methyl-β-D-galactopyranoside	–	–
D-(+)-Glucosamine	–	+/–
D-(–)-Galactosamine	–	+/–
D-(+)-Lactose	–	–
Sucrose	+ +	+
Cellobiose	–	+
Mannitol	–	+
D-(–)-Sorbitol	–	–

[a] Concentration of carbon sources was 10 mM.

[b] GUS activities above 10^{-10} were considered to be induced (+); values below this threshold were not induced (–). For each carbon source, at least two independent experiments were performed upon two different samples in each case. GUS activities were determined 14 hr postinoculation.

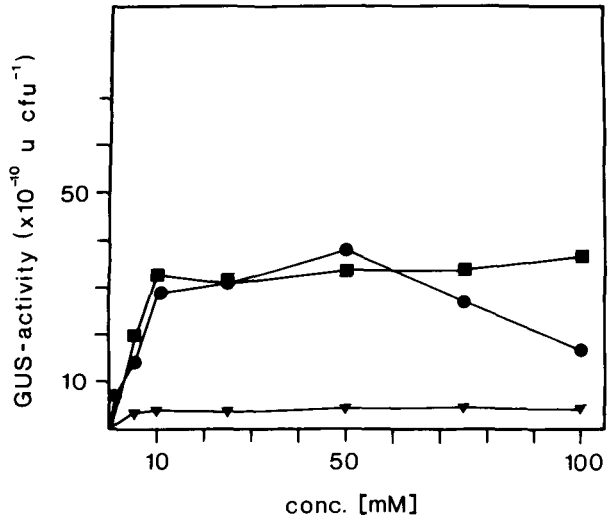

Figure 2. Effects of Different Concentrations of Inducing Sugars on *hrpF* Expression.

GUS activity of the *hrpF-gusA* fusion in strain 85-10 (pF312) was determined after bacterial growth for 14 hr in modified MM1 medium (20 mM NaCl; 0.5 mM phosphate), supplemented with methionine (2 μg/mL) and different concentrations of sucrose (■), fructose (●), or glucose (▼). Units were calculated as described in Methods. The results are the mean of two independent experiments performed upon two different samples in each case.

XVM1 containing different carbon sources. Each carbon source was also tested alone in modified MM1 containing methionine but no sucrose. The results are summarized in Table 3. Addition of pyruvate, succinate, glutamine, or sodium citrate suppressed sucrose induction. When sucrose was replaced by one of these compounds, no induction was observed. Addition of other carbohydrates, e.g., glucose, glycerol, mannitol, or myo-inositol, did not suppress *hrpF* induction. Addition of fructose to XVM1 induced *hrpF* to even higher levels than sucrose alone.

Effect of pH and Sodium Chloride on Induction

Because gene induction is dependent on low pH in other plant pathogenic bacteria, the effects of pH on induction of the *hrpF-gusA* fusion were tested. The pH of XVM1 was pH 6.7. The results for induction assays of *hrpF* in XVM1 medium adjusted to pH 5 to 8 are shown in Figure 3. Optimal induction occurred at pH 6.5 to 7.5. At pH 8, induction was completely inhibited although bacterial growth was not affected. The pH of TCM was pH 5.7.

To test the effects of changes in osmolarity on *hrpF* induction, the concentration of NaCl, normally 20 mM, was increased up to 100 mM. The results are shown in Figure 4. Induction levels of *hrpF* were inversely correlated with increasing concentrations of NaCl. For example, in 100 mM NaCl, induction is only 10% of the value reached in XVM1 containing 20 mM NaCl; cell growth was not affected under these conditions.

Inducibility of *hrpF* and Other *hrp* Loci of *X. c. vesicatoria* in Different Genetic Backgrounds

To test inducibility of other *hrp* loci besides *hrpF* in XVM1, the Tn*3-gus* insertion derivatives pA14, pB35, pC52, pD54, and pF312, representing inducible transcriptional fusions between *hrpA, hrpB, hrpC, hrpD,* and *hrpF* and the *gusA* gene (Schulte and Bonas, 1992), were introduced into *X. c. vesicatoria* strain 85-10, into *X. c. campestris* strain 1147, and into *X. campestris* strain T55 by triparental matings. GUS activities of these merodiploid strains after growth in XVM1 are summarized in Table 4. In strain 85-10, induction of *hrp* loci other than *hrpF* did not reach levels above 0.3 to 0.9 × 10^{-10} units per cfu, indicating that they are not inducible in XVM1. By contrast, in TCM these fusions produced threefold to 20-fold higher values. Significant induction in XVM1, however, occurred with *hrp-gusA* fusions in the *X. c. campestris* background. Strains carrying pB35, pC52, pD54, and pF312, not induced in complex medium, were induced to levels comparable to those obtained for *X. c. vesicatoria* strain 85-10 after growth in TCM. In *X. c. campestris* strain 1147, only pA14 was not induced in XVM1. When the same plasmids were tested in the opportunistic, nonpathogenic *X. campestris* strain T55, no induction in XVM1 was observed. In T55, the

Table 3. Effect of Different Carbon Sources on *hrpF* Induction in XVM1[a]

	GUS Activity (× 10^{-10} units cfu^{-1})[b]	
Carbon Source[c]	− Sucrose	+ Sucrose[d]
Sucrose	37.0	41.0
Citrate	0.0	0.5
Succinate	0.0	0.3
Pyruvate	0.0	1.3
Glycerol	0.1	37.3
Glucose	3.8	37.0
Fructose	25.5	50.5
Mannitol	0.0	45.1
Myo-inositol	0.0	33.2

[a] Strain 85-10 (pF312), carrying an *hrpF-gusA* fusion, was used to monitor GUS activity.

[b] Units were calculated as described in Methods. The results are the mean of two independent experiments performed on two different samples in each case. GUS activities were determined 14 hr postinoculation.

[c] Carbon sources were used at 10 mM except for glycerol (20 mM) in modified MM1 containing methionine (2 μg/mL).

[d] Carbon sources were added to XVM1.

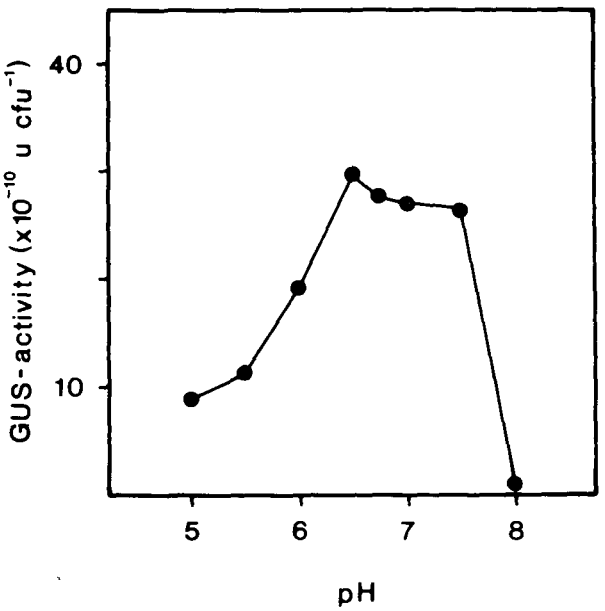

Figure 3. Effect of pH on *hrpF* Induction in XVM1.

Strain 85-10 (pF312) was grown for 14 hr in XVM1 with pH adjusted to different values. Units of GUS activity were calculated as described in Methods. The results are the mean of two independent experiments performed upon two samples in each case.

fusions described above were also not inducible after bacterial growth in the plant or in TCM (Table 4).

DISCUSSION

We have defined a synthetic medium, XVM1, that induces expression of a transcriptional *hrpF-gusA* fusion in *X. c. vesicatoria*. Induction levels were as high as in the plant. The XVM1 medium is a defined medium containing sucrose and L-methionine. Sucrose could be replaced by fructose or by glucose, but induction by glucose was only 10% of the level observed with fructose or sucrose (Figure 2 and Table 2). Besides methionine, cysteine or glutathione was also able to induce *hrpF*, but to lower levels. In contrast to many *Xanthomonas* strains that are auxotrophic for methionine or other amino acids, the *X. c. vesicatoria* strain used in these studies is prototrophic. Therefore, sulfur-containing amino acids seem to have a specific effect on *hrpF* induction. Notably, thiamine, which is also an organic sulfur compound, repressed *hrp* gene induction when added to TCM. A prerequisite for induction by sucrose or fructose and methionine is low concentrations of several components: phosphate, sodium chloride, and organic nitrogen (Figures 1 and 4). Each of these components, including methionine, suppressed the responsiveness of *hrpF* when present in high concentrations. Additionally, the pH of the medium affected inducibility (Figure 3). The optimal pH ranges from 6.5 to 7.5.

It was unexpected that only transcription of *hrpF-gusA* was induced in XVM1, whereas *hrpA-*, *hrpB-*, *hrpC-*, and *hrpD-gusA* fusions were not induced in an *X. c. vesicatoria* wild-type background. Perhaps some component necessary to induce these other loci is missing from XVM1 or present in a suppressing concentration. However, when tested in a different pathovar, *X. c. campestris*, transcription was induced in XVM1 in all cases except for *hrpA* (Table 4). All *hrp* loci were previously shown to be induced in the plant and by bacterial growth in TCM (Schulte and Bonas, 1992), suggesting regulation by a common mechanism. The nature of the regulatory system involved in the expression of *hrp* genes in *X. c. vesicatoria* has not been established. The results presented here indicate that regulation is complex, possibly mediated by several different interacting mechanisms integrating availability of organic and inorganic nutrients and bacterial metabolism. Some of the *hrp* loci were inducible in *X. c. campestris*. Thus, regulation might be different in the two pathovars. This could be explained by differences in the nutritional requirements of *X. c. vesicatoria* and *X. c. campestris*, which, notably, also differ in host tissue specificity (intercellular space and xylem, respectively). In contrast, the regulatory genes required for induction of the *hrp* loci are most likely absent from the nonpathogenic *X. campestris* strain T55. In this strain,

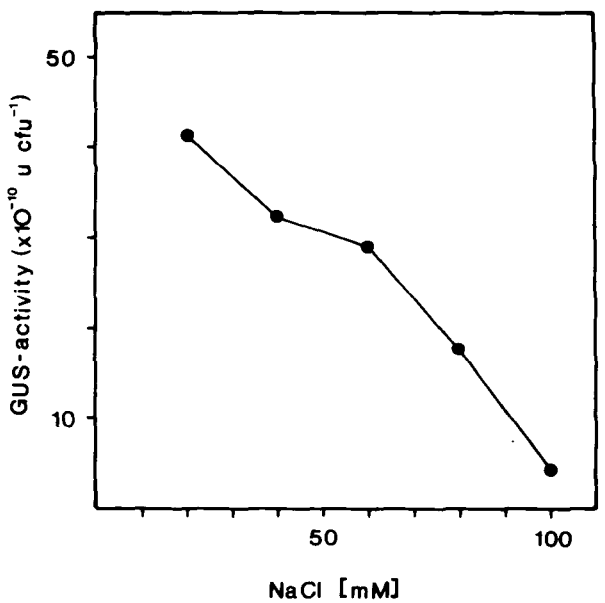

Figure 4. Effects of Different Concentrations of NaCl on *hrpF* Induction.

hrpF Induction was determined 14 hr postinoculation of the cells into XVM1 containing different concentrations of NaCl; XVM1 normally contains 20 mM NaCl. Units of GUS activity were calculated as described in Methods.

Table 4. Expression Levels of *hrp-gusA* Fusions in Different Genetic Backgrounds

		GUS Activity ($\times 10^{-10}$ units cfu^{-1})[b]				
Strain[a]	Medium	pA14	pB35	pC52	pD54	pF312
Xcv 85-10	TCM	7.0	3.0	6.0	7.0	100.0
Xcv 85-10	XVM1	0.3	0.9	0.8	0.3	33.9
Xcc 1147	XVM1	0.7	3.7	4.9	2.0	68.6
Xc T55	XVM1	0.2	0.1	0.1	0.1	0.3
Xc T55	TCM	0.2	0.0	0.0	0.0	0.4

[a] Merodiploid strains; the plasmids pA14, pB35, pC52, pD54, and pF312 carry transcriptional fusions of the *hrp* loci *hrpA*, *hrpB*, *hrpC*, *hrpD*, and *hrpF*, respectively, to the *gusA* gene (see Methods).
[b] Units are calculated as described in Methods. Values are averages of three independent experiments, performed on two different samples in each case.

none of the transcriptional fusions was induced under any condition tested. It was established previously that in strain T55 presumably a large DNA region, including the *hrp* cluster, is missing (Bonas et al., 1991).

Plant-independent activation of *hrp* genes has been described before. However, the finding that expression of an *hrp* locus is dependent on two different organic compounds and phosphate starvation is novel. Fellay et al. (1991) stated induction of *hrp* genes from *P. s. phaseolicola* in M9 minimal medium but did not report the carbon source used. Beer et al. (1991) reported that in *Erwinia amylovora*, *hrp* genes were repressed in rich medium and induced in a minimal medium containing mannitol. The induction medium previously described to induce the avirulence gene *avrB* and *hrp* genes in pathovars of *P. syringae* (Huynh et al., 1989; Huang et al., 1991) did not induce *hrpF* in *X. c. vesicatoria* (R. Schulte, unpublished data). This was most likely due to the higher amount of phosphate (50 mM) present in that medium. The carbohydrates optimal for induction of genes in *P. s. glycinea* were fructose, sucrose, and mannitol (Huynh et al., 1989). Mannitol had no effect on induction of *hrpF* in *X. c. vesicatoria*. Recently, expression studies of *hrp* genes in *X. c. campestris* were reported (Kamoun and Kado, 1990; Arlat et al., 1991). As in *X. c. vesicatoria*, the *hrp* genes of *X. c. campestris* studied are repressed in complex medium but induced in minimal medium (Arlat et al., 1991). The carbon sources optimal for induction were sucrose, glutamate, and glycerol. In *X. c. vesicatoria*, glycerol did not have a positive effect; glutamate was not tested. The minimal medium used in *X. c. campestris* (Arlat et al., 1991) contained casamino acids as organic nitrogen source, higher concentrations of which suppressed gene induction (as was observed for *hrpF* in *X. c. vesicatoria*). The same effect was also reported by Arlat et al. (1990) for *hrp* gene expression in *P. solanacearum*.

Given that both *P. s. glycinea* and *X. c. vesicatoria* multiply in the intercellular space of the plant leaf, the fact that fructose or sucrose is needed for *hrp* induction might reflect the nutritional conditions available, as has been suggested previously (Huynh et al., 1989). It is conceivable also that other components of XVM1, e.g., phosphate and methionine, are present in the intercellular space of the plant, possibly in similar concentrations. Methionine, besides other amino acids, has been found in the intercellular space of cotton cotyledons (M.L. Ziegler and M. Essenberg, personal communication). Preliminary data indicate that in XVM1 without any added phosphate there is no induction of *hrpF* in *X. c. vesicatoria* (R. Schulte, unpublished results).

Although the media found for induction of *hrp* genes differ in the systems studied so far, they have some features in common. First, all media are minimal salt media to which a particular carbon source has to be added. Sucrose seems to be a good inducer in several systems. Organic nitrogen, required for *hrpF* induction, is not present in the medium described by Huynh et al. (1989). For *X. c. campestris*, it was provided in the form of casamino acids (Arlat et al., 1991); however, it is not known whether it is necessary for induction. Interestingly, addition of TCA intermediates (e.g., succinate, citrate) to the respective inducing medium has been found to suppress gene induction in *P. s. glycinea* (Huynh et al., 1989), *X. c. campestris* (Arlat et al., 1991), and *X. c. vesicatoria* (Table 3). This was interpreted by Huynh et al. (1989) as evidence for catabolite repression and could also explain the results obtained with *X. c. vesicatoria*.

The effect of osmolarity on *hrp* gene induction has not been well studied. In *X. c. vesicatoria*, *hrpF* induction was not significantly reduced in the presence of increasing amounts of an inducing carbon source (Figure 2). However, increases in ionic strength by the addition of NaCl clearly had a negative effect on induction. The suppressing effect by NaCl was also reported by Fellay et al. (1991) but was not observed by Beer et al. (1991) in *Erwinia amylovora*.

In the aforementioned systems, an effect of phosphate on gene induction has not been described. In *X. c. vesicatoria*, however, addition of high amounts of phosphate to TCM suppressed *hrp* induction. This was also observed when the phosphate concentration of XVM1, normally 0.5 mM, was increased 10- to 20-fold (R. Schulte, unpublished results). Phosphate starvation plays an important role in the induction of a number of genes in different prokaryotes. For example, in *Escherichia coli*, genes involved in phosphate uptake and metabolism are induced at low phosphate levels (Wanner, 1987). One might speculate that in *X. c. vesicatoria* phosphate starvation initiates a cascade of control functions involving a so far unknown one- or two-component regulatory system that activates *hrpF*. The only example known for gene induction by phosphate starvation in a phytopathogenic bacterium is *virG* of *Agrobacterium tumefaciens* (Winans et al., 1988). As with *virG* in *A. tumefaciens* (Winans et al., 1988) and *avrB* in *P. s. glycinea* (Huynh et al., 1989), induction of *hrpF* in XVM1 was pH dependent; however, the pH optimum in the other systems was lower.

Although the biochemical function of *hrp* genes and the nature of the corresponding regulatory genes remain to be elu-

cidated, we have shown here that at least some *hrp* genes can be induced in a synthetic medium without any plant-derived factor. In view of our difficulties purifying the inducing factor from TCM, it was perhaps not surprising to find the necessity for two different organic components that have to be present simultaneously for *hrp* gene induction. It cannot be ruled out, however, that there are additional, plant-specific molecules present in TCM that may play a role in the induction of the *hrp* loci that are not induced in XVM1. Whether the XVM1 medium reflects the conditions available to the bacteria in the intercellular space is a matter of speculation. One possible function of the genes encoded in *hrpF* might be that they are part of a transport system for sucrose or fructose and/or methionine. However, the finding that the marker exchange mutants in *hrpA* to *hrpF* are still able to grow on agarose plates containing sucrose or fructose, irrespective of the presence of methionine, may argue against this hypothesis (R. Schulte, unpublished results). On the other hand, bacteria often have multiple transport systems that might substitute each other (Halpern, 1974). It is anticipated that further studies, including measurement of transport of the organic inducers into the bacterium, will help to gain more information about function and regulation of *hrp* gene expression.

METHODS

Bacterial Strains, Plasmids, and Media

The *Xanthomonas campestris* pv *vesicatoria* strains used were as follows: 85-10, wild type (Minsavage et al., 1990); and strain 85-10::hrpF312, which is a marker exchange mutant carrying a Tn*3-gus* insertion and exhibiting a null phenotype (Bonas et al., 1991). The *X. c.* pv *campestris* strain NCPPB 1147 is pathogenic on radish and was obtained from Dr. M.J. Daniels. *X. campestris* strain T55 is opportunistic and nonpathogenic (Bonas et al., 1991; obtained from Dr. R.E. Stall). Plasmids pA14, pB35, pC52, pD54, and pF312 are Tn*3-gus* derivatives of cosmids containing the cloned *hrp* region of *X. c. vesicatoria* (Bonas et al., 1991; Schulte and Bonas, 1992). The transposon Tn*3-gus* carries a promoterless β-glucuronidase (*gusA*) gene (Bonas et al., 1989).

Plasmids were introduced into *Xanthomonas* by conjugation using pRK2013 as a helper plasmid in triparental matings (Figurski and Helinski, 1979; Ditta et al., 1980). Strains of *Escherichia coli* were cultivated in Luria-Bertani medium (Miller, 1972). *Xanthomonas* strains were routinely grown at 28°C in NYG broth (Turner et al., 1984) or on NYG 1.5% agar. The minimal media used were M9 (Miller, 1972), MM1 (Roy et al., 1988), or MS medium (Murashige and Skoog, 1962), the latter supplemented with 2,4-dichlorophenoxy acetic acid (1 μg/mL). The M9 medium used in our studies also contained thiamine (10 μg/mL). The minimal media were supplemented with different carbohydrates and organic nitrogen sources; casamino acids were from Difco (see text). The original MM1 contained the following components: 100 mM NaCl, 10 mM $(NH_4)_2SO_4$, 5 mM $MgSO_4$, 1 mM $CaCl_2$, 8 mM KH_2PO_4, 16 mM K_2HPO_4, and micronutrients. For all of our studies using MM1, the concentration of NaCl was reduced to 20 mM. Other modifications are described in Results.

Amino acids were added to 2 μg/mL, carbon sources to 10 mM, unless otherwise stated. Antibiotics were added to the media at the following final concentrations: kanamycin, 50 μg/mL; tetracycline, 10 μg/mL; rifampicin, 100 μg/mL.

Preparation of TCM

Callus suspension cell lines of tomato cv Money Maker were grown in MS medium, supplemented with 2% sucrose and 2,4-dichlorophenoxy acetic acid (1 μg/mL). The 250-mL flasks containing 50 mL suspension were incubated at 27°C on a shaker at 110 rpm for 7 days before 10% of the suspension was subcultured by dilution into fresh medium.

To obtain TCM, the cell-free filtrate of a 7-day-old suspension culture was filtered through 0.22 μm nitrocellulose and stored at −80°C.

Assay of GUS Activity

For GUS assays, the bacteria were grown in NYG medium (Turner et al., 1984) overnight, collected by centrifugation, and washed twice in 1 mM $MgCl_2$ before inoculation into a particular minimal medium. After 14 hr, cells were harvested by centrifugation and resuspended in assay buffer (Jefferson et al., 1987). Aliquots were taken for the enzyme assay. The number of bacteria (cfu) per assay were calculated by plating appropriate dilutions on selective medium. GUS activity was determined in fluorometric assays using 4-methylumbelliferyl glucuronide as substrate as described (Jefferson et al., 1987). One unit of GUS is defined by the amount of nanomoles of 4-methylumbelliferone released per minute.

Assays of GUS activity of bacteria grown on solid minimal medium were performed as follows. Bacteria were grown overnight in NYG broth, harvested by centrifugation, and washed twice in 1 mM $MgCl_2$ before inoculation into XVM1. After a preincubation period of 16 hr, appropriate dilutions were plated onto agarose plates containing modified MM1 with methionine and different carbon sources. To stain for GUS activity of bacterial colonies, they were overlaid with a solution of the assay buffer (see above) that was diluted 10-fold in water and contained 4-methylumbelliferyl glucuronide as substrate (Jefferson et al., 1987). Fluorescent colonies were visible on a UV-light transilluminator.

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