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HrpG, a Key *hrp* Regulatory Protein of *Xanthomonas campestris* pv. *vesicatoria* Is Homologous to Two-Component Response Regulators

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***Xanthomonas campestris* pv. *vesicatoria* is the causal agent of bacterial spot disease of pepper and tomato plants. Expression of its basic pathogenicity genes, the *hrp* genes, is induced in planta and in XVM2 medium and is dependent on the *hrp* regulatory gene *hrpXv* for five out of six loci in the 23-kb *hrp* cluster. Here we describe the isolation of a novel *hrp* gene, *hrpG*, that was identified after chemical mutagenesis and that is located next to the *hrpXv* gene. In a *hrpG* mutant induction of expression of the seven loci *hrpA* to *hrpF*, and *hrpXv* is abolished, suggesting that *hrpG* functions at the top of the *hrp* gene regulatory cascade. *hrpG* is the only gene in the locus and encodes a putative protein of 263 amino acids with a molecular mass of 28.9 kDa. The HrpG amino acid sequence shows similarity to response regulator proteins of the OmpR subclass of two-component systems, being mostly related to the ChvI proteins of *Agrobacterium tumefaciens* and *Rhizobium* spp., and TctD of *Salmonella typhimurium*. Expression of *hrpG* is low in complex medium, is increased in XVM2 by a factor of four, and is independent of other *hrp* loci. A model on *hrp* gene regulation in *Xanthomonas campestris* pv. *vesicatoria* is discussed.**

Additional keywords: sms gene, type III secretion.

Xanthomonas, *Pseudomonas*, *Burkholderia*, and *Erwinia* are major genera of gram-negative phytopathogenic bacteria that infect almost all crop plants and are responsible for important economic losses. Although disease symptoms caused by these bacteria are very diverse, pathogens of all genera share a common feature: They contain a large cluster of basic pathogenicity genes, the *hrp* genes (hypersensitive reaction and pathogenicity) (reviewed in Bonas 1994; Willis et al. 1991). *Xanthomonas campestris* pv. *vesicatoria* is a nonsystemic pathogen that causes bacterial spot disease on pepper and tomato. Its 23-kb *hrp* cluster comprises six *hrp* loci, *hrpA* to *hrpF*, which are all required for full pathogenicity (Bonas et al. 1991). *hrp* mutant bacteria no longer grow in the leaf intercellular space, provoke no disease symptoms in a susceptible plant, and do not elicit the hypersensitive reaction (HR) on a resistant host or nonhost plant. Based on sequence analysis (U. Bonas, unpublished; Fenselau et al. 1992; Fenselau and Bonas 1995; Wengelnik et al. 1996) 21 proteins are predicted to be encoded in the *hrp*-cluster of *X. campestris* pv. *vesicatoria*, 10 of which show significant homology to Hrp proteins in *P. syringae*, *E. amylovora*, and *B. solanacearum* (formerly *P. solanacearum*, now also referred to as *Ralstonia solanacearum*) (Bogdanove et al. 1996). These conserved genes are also related to pathogenicity genes of *Yersinia* and *Shigella* that are involved in type III secretion of proteinaceous virulence factors (Forsberg et al. 1994; Van Gijsegem et al. 1993) and thus indicated a role of *hrp* genes in protein secretion (Fenselau et al. 1992; Gough et al. 1992). Hrp-dependent secretion has indeed been demonstrated (Arlat et al. 1994; He et al. 1993; Wei et al. 1992).

Transcriptional regulation of *hrp* genes depends on environmental conditions. *hrp* gene expression is generally suppressed in complex media and induced in planta and under certain in vitro conditions (Bonas 1994; Rahme et al. 1991; Wei et al. 1992; Xiao et al. 1992). In *X. campestris* pv. *vesicatoria*, *hrp* gene expression has been studied using transcriptional *hrp-gusA* fusions and was found to be induced in planta, in tomato-conditioned medium TCM (Schulte and Bonas 1992a), and in synthetic XVM2 medium (Wengelnik et al. 1996). Expression is suppressed in complex medium except for that of *hrpE* that shows a basal level of expression which is increased by growth in XVM2 medium (Wengelnik and Bonas 1996). In contrast to *E. amylovora* (Wei and Beer 1995), *P. syringae* (Grimm and Panopoulos 1989; Xiao et al. 1994), and *B. solanacearum* (Genin et al. 1992) the *hrp* regulatory genes of *X. campestris* pv. *vesicatoria* are located outside of the large *hrp* cluster and its flanking regions. *hrpXv*, which codes for a protein of the AraC family, has been shown to be indispensable for transcriptional activation of the five loci *hrpB* to *hrpF* (Wengelnik and Bonas 1996). However, induced expression of the *hrpA* locus and the *hrpXv* gene itself is independent of HrpXv (Wengelnik and Bonas 1996; Wengelnik et al. 1996), suggesting the presence of at least one additional regulatory gene.

In this paper we describe the isolation of a novel *X. campestris* pv. *vesicatoria hrp* gene, *hrpG*, that is required for transcriptional activation of all *hrp* genes so far isolated. HrpG is proposed to be part of a two-component system in which it functions as a response regulator.

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RESULTS

Identification of the *X. campestris* pv. *vesicatoria hrpG* locus.

We isolated a novel, NTG induced *hrp* mutant (E2-12) of *X. campestris* pv. *vesicatoria* 85E that failed to cause disease in a susceptible plant and did not induce the HR in resistant plants. Mutant E2-12 could not be complemented with both the large *hrp* gene cluster (*hrpA* to *hrpF*) and by the *hrp* activator gene *hrpXv*. We therefore tried to isolate a complementing cosmid from the genomic library of *X. campestris* pv. *vesicatoria* 75-3. The library was conjugated into mutant E2-12, transconjugants were collected, and infiltrated into leaves of the susceptible pepper cultivar ECW. One week after inoculation water-soaking spots appeared, the typical disease symptoms, from which bacteria were reisolated. Two different cosmids were identified which contained nearly identical inserts and which both were able to complement mutant E2-12. The cosmid with the larger insert, pXV1, was chosen for further studies. The novel *hrp* locus affected in mutant E2-12 was designated *hrpG*.

Genetic and transcriptional analysis of the *hrpG* region.

Cosmid pXV1 was mutagenized using Tn*3-gus* to genetically characterize the *hrpG* region. Fifty-six transposon derivatives of pXV1 were conjugated into *X. campestris* pv. *vesicatoria* mutant E2-12 to test for complementation. All insertion derivatives restored the mutant except for one, 1006. Restriction analysis revealed that insertion 1006 was located 3 kb from the left end of the pXV1 insert (Fig. 1). Insertion 1006 and two other transposon insertions, 1013 and 1016, that mapped to the vicinity of the insertion site of 1006 were introduced into *X. campestris* pv. *vesicatoria* 85-10 by marker exchange mutagenesis. The resulting genomic mutants were tested for their phenotype in planta. While strains 85-10::1013 and 85-10::1016 behaved like the parental wild-type strain, the marker exchange mutant 85-10::1006 showed a typical *hrp* phenotype, i.e., failed to cause water-soaking symptoms and to multiply in the susceptible pepper line ECW, and did not elicit the HR in resistant pepper line ECW-10R (data not shown). This mutant, designated 85G, is prototrophic and could be complemented for pathogenicity by plasmid pXV1. To determine the minimal DNA region needed for *hrpG* function, DNA fragments from pXV1 were subcloned into pLAFR vectors and introduced into mutant 85G. pSG72, pSG73, and pSG74 which contain 1.7, 1.3, and 1.2-kb inserts, respectively, in pL6GUSB (Fig. 1) all complemented mutant 85G while the 2.5-kb *Hin*dIII (polylinker site)-*Eco*RI fragment in pLG75 did not. It should be noted that strain 85G(pSG74) carrying the shortest insert caused symptoms that appeared later than with wild-type strain 85-10; HR on a resistant pepper line was delayed by 6 h and watersoaking on a susceptible line by 1 to 2 days. We concluded that *hrpG* including its promoter is located on a 1,330-bp *Xba*I-*Kpn*I fragment present in pSG73.

hrpG functions as a key regulatory gene of *X. campestris* pv. *vesicatoria hrp* genes.

Previous studies on regulation of the *hrp* gene cluster have revealed that the *hrpXv* gene is involved in transcriptional activation of most, but not all *hrp* loci: Expression of *hrpA* and *hrpXv* itself is independent of the *hrpXv* gene (Wengelnik and Bonas 1996; Wengelnik et al. 1996). We therefore analyzed whether the novel *hrpG* gene plays a role in *hrp* gene regulation. For this purpose we used *hrp*-promoter-*gusA* fusions that resulted from Tn*3-gus* insertions in the loci *hrpA* to *hrpF* (pXV9::A14, pXV9::B35, pXV9::C17, pXV9::D54, pXV4::E525, and pXV2::F312) and an *hrpXv*-promoter-*gusA* fusion (pPX2). These plasmids were introduced into mutant 85G and wild-type 85-10. Mutant 85G was suitable for these experiments since it showed no detectable GUS activity, although it carries a *gusA* gene inserted in *hrpG* suggesting that orientation of *hrpG* transcription is probably opposite to that of the *gusA* gene. GUS activities of transconjugants were measured after growth in complex medium NYG and *hrp* inducing medium XVM2 (Fig. 2). In the *hrpG* mutant, all seven

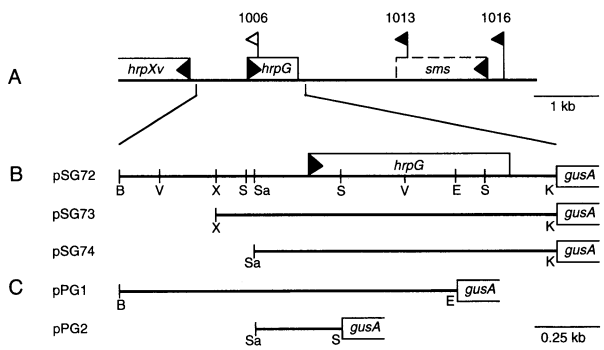

Fig. 1. Genetic organization and restriction map of the *hrpG* region. **A,** Location and orientation of *hrpG*, *hrpXv*, and the hypothetical *sms* gene are shown as open bars; the black arrowheads indicate direction of transcription. The position of Tn*3-gus* insertions 1006, 1013, and 1016 are indicated and the orientation of the *gusA* gene (from the right to the left) is given by the triangles; filled triangles correspond to GUS active, the open triangle to GUS inactive insertions. The region shown corresponds to the insert of plasmid pBG1. **B,** Restriction map of *hrpG* and subclones used in this study. Fusion to the promoterless *gusA* gene in pL6GUSB is indicated. **C,** *hrpG* promoter-*gusA* fusions in pL6GUSB. Relevant restriction sites are, B, *Bam*HI; E, *Eco*RI; K, *Kpn*I; S, *Sac*I; Sa, *Sal*I; V, *Eco*RV; X, *Xba*I.

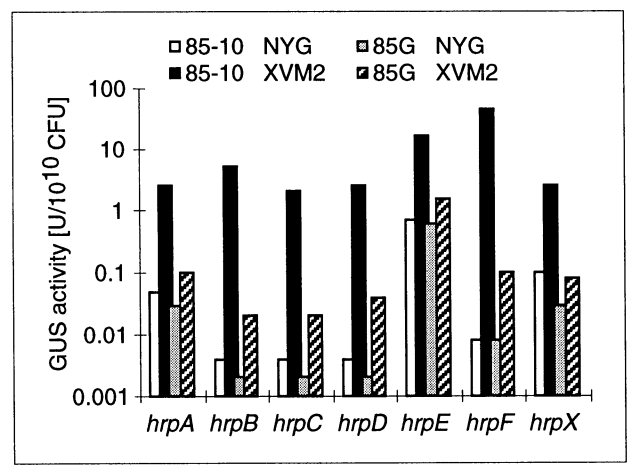

Fig. 2. Expression of different *hrp* loci in the wild type and the *hrpG* mutant. Wild type 85-10 and the *hrpG* mutant 85G carrying plasmid borne Tn*3-gus* transcriptional fusions in the loci *hrpA* to *hrpF*, and an *hrpXv*-promoter-*gusA* fusion (see text for details) were grown for 14 to 16 h in NYG and XVM2. Specific GUS activities are the average of two experiments and are graphically displayed using a logarithmic scale. Values below 0.01 U/10^{10} CFU are considered as background activity.

hrp promoters tested showed strongly reduced GUS activities in XVM2 as compared to those in the wild type. In NYG, the values were about the same for 85G and 85-10. In a control experiment, we measured strong GUS activity in 85G carrying the *gusA* gene under control of the *lacZ* promoter (p*lacZ* is constitutively active in *Xanthomonas*) showing that function of GUS is not affected in 85G (data not shown). Thus, a functional *hrpG* gene is indispensable for transcriptional activation of the *hrp* loci *hrpA* to *hrpF* and the regulatory gene *hrpXv*.

Sequence analysis of *hrpG*.

The 1.7-kb region from the *Bam*HI to the *Kpn*I site (Fig. 1) which has been shown to be sufficient for *hrpG* function (see above) was sequenced. Figure 3 shows the sequence starting from the *Sal*I site. A 789-nucleotide open reading frame (ORF) with high probability of expression as analyzed by 'Codonpreference' was identified and designated *hrpG*. The predicted translation initiation codon (ATG) is at position 215 and is preceded by a putative ribosome binding site. With the translation stop codon at position 1004 *hrpG* is predicted to encode a protein (HrpG) of 263 amino acids and a molecular mass of 28.9 kDa. HrpG is hydrophilic and has an isoelectric point of 7.3. The determination of the exact site of transposon insertion 1006 confirmed that the orientation of the *gusA* gene is inverse to transcription of *hrpG*. The transposon is inserted after nucleotide 485 as shown in Figure 3, thus interrupting translation of HrpG after amino acid 57. Inspection of the sequence did not reveal homology to rho-independent transcription terminators. Nevertheless, transcription appears to be terminated immediately downstream of *hrpG*. N-terminal fusions of the *gusA* gene to the *Kpn*I site 166-bp downstream of the *hrpG* stop codon in plasmids pSG72, pSG73, and pSG74 (see Fig. 1) did not result in detectable GUS activities when these plasmids were introduced into wild-type strain 85-10. Thus, *hrpG* seems to be the only gene in the locus. Inspection of the *hrpG* promoter region did not reveal any similarities to other *Xanthomonas* promoters or to common promoter sequences described for *Escherichia coli*.

We found that the 163-bp sequence from the *Bam*HI to the *Eco*RV site is the reverse complement of the published *hrpXv* promoter sequence (Wengelnik and Bonas 1996). Hence, the two regulatory genes *hrpG* and *hrpXv* are located next to each other and are divergently transcribed. The distance between the two translation initiation codons is 829 nucleotides. Restriction analysis of plasmid pXV1 suggested that it might be identical to the previously isolated plasmid pXV751 (Wengelnik and Bonas 1996).

We analyzed whether the region downstream of *hrpG* also contains interesting ORFs. The sequence of 600 bp immediately downstream of the *Kpn*I site (see Fig. 1) shows no significant similarity to any sequence in the databases. In contrast, an incomplete ORF approximately 2 kb downstream of *hrpG* encodes a 264 amino acid peptide that is 62% identical to the *E. coli* Sms protein (sensitivity to methylmethane sulfonate) (Neuwald et al. 1992). The constitutively expressed *E. coli sms* gene encodes a protein of 460 amino acids. In *E. coli*, a mutation in the *sms* gene leads to increased sensitivity to alkylating agents, and the *sms* gene is therefore suggested to be involved in DNA repair after DNA damage by alkylation (Neuwald et al. 1992). We found that the Tn*3-gus* insertions 1013 and 1016 that map to this region (see Fig. 1) constitutively express GUS (1 and 7 U/10^{10} CFU, respectively). This corroborates the predicted direction of transcription and might reflect expression of the *sms* homologous gene.

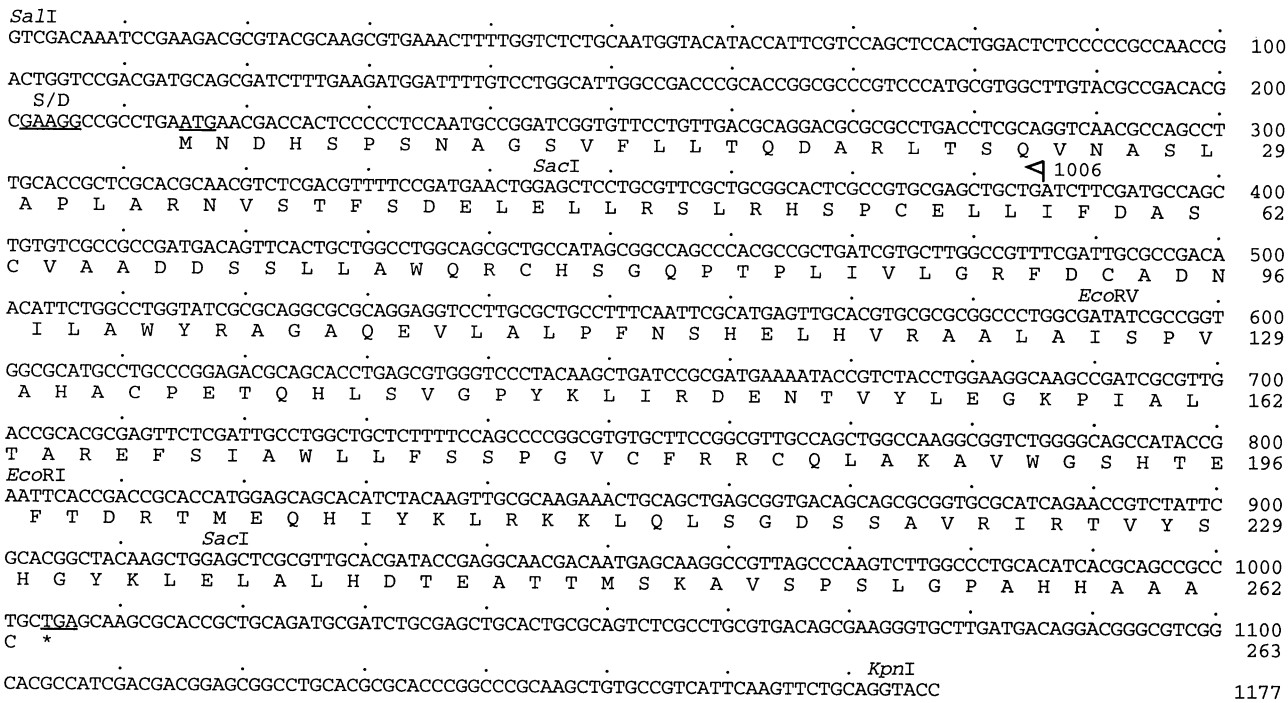

Fig. 3. Nucleotide sequence of *hrpG*. The 1,177-bp DNA sequence from the *Sal*I- to the *Kpn*I site is shown. The translation product of HrpG is given in the one-letter-code. Translational start and stop codons of HrpG and a putative Shine-Dalgarno sequence (S/D) are underlined. Relevant restriction sites indicated. Tn*3-gus* insertion 1006 is inserted after nucleotide 385.

HrpG shows homology to response regulator proteins of two-component systems.

Searches in the databases revealed that HrpG is related to response regulator proteins of so-called 'two-component' regulatory protein pairs (Albright et al. 1989; Parkinson and Kofoid 1992). HrpG is most similar to the ChvI proteins of *Agrobacterium tumefaciens* (Charles and Nester 1993), and *Rhizobium* spp. (Osteras et al. 1995), TctD of *Salmonella typhimurium* (Widenhorn et al. 1989), and to PhoB proteins that have been isolated, for example, from *Escherichia coli* (Makino et al. 1986), *Shigella flexneri* (Scholten et al. 1995), *Rhizobium meliloti* (McLean et al. 1994), and *Klebsiella oxytoca* (Lee et al. 1989). These proteins to which HrpG shows up to 51% similarity and 29% identity have been grouped into a subclass of response regulators that includes the well-studied *E. coli* OmpR and *A. tumefaciens* VirG proteins. The multiple sequence alignment in Figure 4 reveals that sequence conservation is significantly higher in the C-terminal 110 amino acids of HrpG (up to 61% similarity and 40% identity) than in the N-terminal 150 amino acids (maximal 44% similarity and 27% identity). These two domains of HrpG also differ remarkably in their predicted isoelectric points of 5.5 and 10.2 for the N- and C-terminal domains, respectively.

***hrpG* is expressed in complex medium.**

The complementation experiments presented above showed that plasmid pSG72 fully complemented the *hrpG* mutant 85G while complementation was only partial with pSG74. We assumed that pSG72 would contain the complete *hrpG* promoter and cloned appropriate fragments of pSG72 and pSG74 into promoter test plasmid pL6GUSB. The resulting plasmids pPG1 and pPG2 (Figure 1), containing 753 and 214 bp, respectively, upstream of the predicted *hrpG* translation initiation codon, were introduced into wild-type strain 85-10. GUS activities were determined after growth in NYG and XVM2 medium (Fig. 5). pPG1 expressed GUS at low level in NYG (0.7 U/10^{10} CFU) while in XVM2 expression was induced three- to fourfold (2.4 U/10^{10} CFU). The shorter construct, pPG2, showed very low GUS activity in NYG in the range of background (0.04 U/10^{10} CFU). Induction in XVM2 was 10-fold (0.4 U/10^{10} CFU). These results indicated that pPG2 lacked sequences needed for full activity of the *hrpG* promoter. It is therefore likely that the partial complementation observed with plasmid pSG74 was due to weak expression of *hrpG* in this plasmid.

To analyze whether autoregulation or feedback regulation is involved, GUS activities of pPG1 and pPG2 were measured in the *hrp* mutants 85G (*hrpG*), 85EX (*hrpXv*), and 85ΔAD (*ΔhrpA1* to *hrpD4*) (Fig. 5). The values obtained for pPG2 were in the same range in all four strains, indicating that expression is independent of the chromosomal background. For pPG1, expression was similar in 85-10, 85EX, and 85ΔAD while two- to threefold higher values were obtained in the *hrpG* mutant. This result indicates that no feedback, but nega-

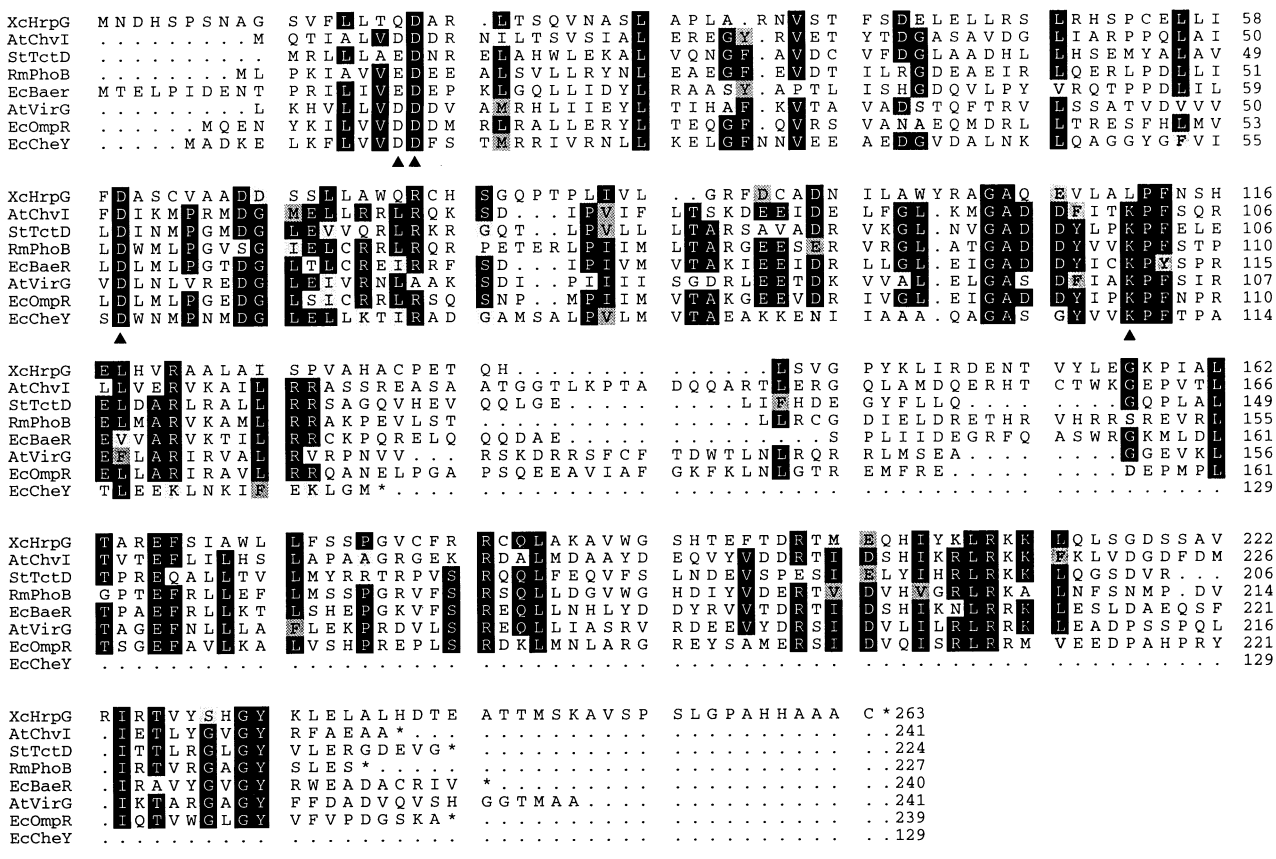

Fig. 4. Alignment of the HrpG peptide sequence with its homologs ChvI (Charles and Nester 1993) and VirG (Winans et al. 1986) of *Agrobacterium tumefaciens*, TctD of *Salmonella typhimurium* (Widenhorn et al. 1989), PhoB of *Rhizobium meliloti* (McLean et al. 1994), BaeR (Nagasawa et al. 1993), OmpR (Wurtzel et al. 1982), and CheY (Mutoh and Simon 1986) of *E. coli*. Amino acids highlighted in black are identical in more than four proteins and shadings in grey correspond to conserved residues. Critical amino acid positions for CheY function are marked with a triangle.

tive autoregulation might affect *hrpG* promoter activity and that sequences involved in this repression are missing in pPG2.

DISCUSSION

In this study we report on the isolation and characterization of *hrpG*, a novel *hrp* locus of *X. campestris* pv. *vesicatoria*. *hrpG* was found to play a key role in *hrp* gene regulation, since a *hrpG* mutation no longer allows induction of all other *hrp* loci identified so far (*hrpA*, *B*, *C*, *D*, *E*, *F*, and *hrpX*) under *hrp* gene inducing conditions. Interestingly, *hrpG* and the previously described *hrp* regulatory gene *hrpXv* (Wengelnik and Bonas 1996) are located outside of the large *hrp* gene cluster but next to each other. The two genes are transcribed from a divergent promoter region. The *hrpG* promoter shows a basal level of expression in complex medium and its activity is only slightly enhanced in XVM2. Factors and sequence motifs responsible for these effects are not known.

hrpG encodes a protein with significant sequence similarity to response regulator proteins of bacterial two-component systems. These regulatory systems have been found in many prokaryotic species and play a role in adaptation of the bacterial cell to changing environmental conditions. It has been estimated that *E. coli* contains 40 to 50 different sensor/regulator pairs (Parkinson and Kofoid 1992). The 'sensor protein', often associated with the inner membrane, functions as a histidine kinase that autophosphorylates upon stimulation and transphosphorylates its cognate response regulator protein on an N-terminal aspartate thereby changing its activation status (reviewed in Albright et al. 1989; Parkinson and Kofoid 1992). The sensor and regulator proteins have been classified into subgroups depending on their predicted domain structure (Parkinson and Kofoid 1992). Accordingly, HrpG belongs to the subgroup containing, for example, the *E. coli* proteins OmpR (Comeau et al. 1985; Wurtzel et al. 1982) and PhoB (Makino et al. 1986), *S. typhimurium* PhoP (Miller et al. 1989) and TctD (Widenhorn et al. 1989), and *A. tumefaciens* VirG (Winans et al. 1986). These proteins regulate genes playing a role in as different responses as adaptation to osmolarity (OmpR), metabolism of carbon (TctD), and phosphate (PhoB), and virulence (VirG, PhoP). OmpR (Forst et al. 1989), PhoB (Makino et al. 1989), and VirG (Roitsch et al. 1990) have been shown to be phosphorylated, thereby enhancing their binding to specific regulatory sequences involved in transcriptional activation. A sequence alignment of HrpG with its homologs (Fig. 4) revealed highest sequence conservation in the C-terminal domain that characterizes this subgroup of response regulators, and lower conservation for the N-terminal 'receiver' domain. In fact, the N-terminal domains of all aligned proteins show higher sequence similarity to one another than to HrpG. The CheY protein, although completely lacking the C-terminal domain, has been included in Figure 4 because its tertiary structure has been analyzed in detail (Stock et al. 1989; Volz and Matsumura 1991). In CheY, four residues have been shown to play a critical role in phosphorylation and signaling: Asp-12, Asp-13, Asp-57, and Lys-109. The residue that becomes phosphorylated by the sensor protein (Asp-57) (Sanders et al. 1989), lies close to Asp-12 and Asp-13 in an acidic pocket, together with Lys-109. It was suggested that phosphorylation of Asp-57 displaces Lys-109 thereby triggering conformational changes of the protein that lead to alteration of its activity (Parkinson and Kofoid 1992). The Asp-57 of CheY is conserved in HrpG (position 60) suggesting that it might be phosphorylated. The acidic pocket, however, is only partially conserved; the first aspartate is replaced by a glutamine and the lysine is absent, while surrounding sequences are conserved.

The results presented here, together with data obtained recently on the regulatory function of *hrpXv*, led us to propose the following model for *hrp* gene regulation in *X. campestris* pv. *vesicatoria* (Fig. 6). The large *hrp* gene cluster that en-

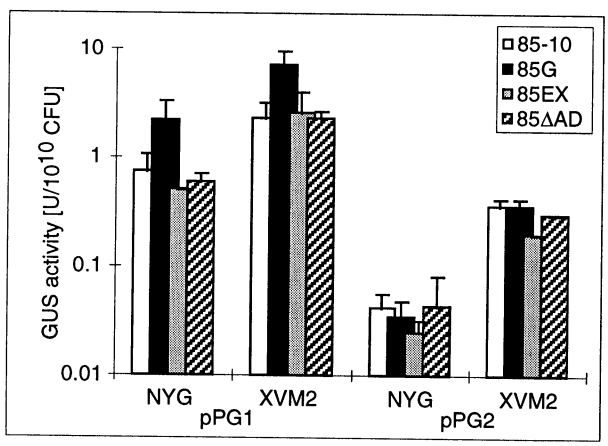

Fig. 5. *hrpG* expression in different genomic backgrounds. The *hrpG*-promoter fusions pPG1 and pPG2 were introduced into wild-type 85-10, *hrpG* mutant 85G, *hrpXv* mutant 85EX, and the *hrpA1* to *hrpD4* deletion mutant 85ΔAD. GUS activities were determined as for Figure 2 and are the average of three experiments. The results are displayed using a logarithmic scale.

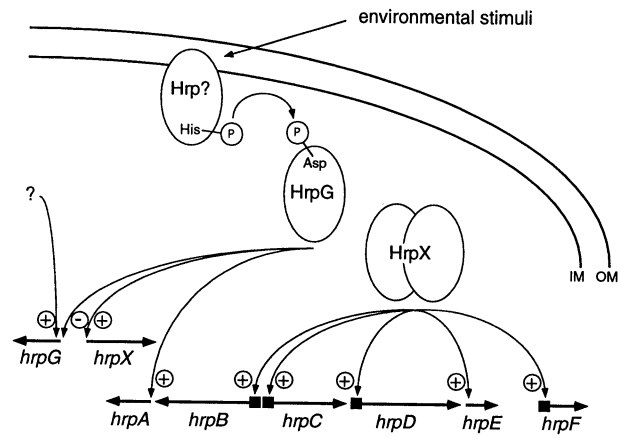

Fig. 6. Model of *hrp* gene regulation in *Xanthomonas campestris* pv. *vesicatoria*. Straight arrows symbolize *hrp* genes and operons and globules represent *hrp* regulating proteins. We propose that an as yet unidentified Hrp protein, which might be localized in the inner membrane, functions as a sensor of *hrp*-inducing stimuli and phosphorylates (P) HrpG on an aspartate. Activated HrpG would then positively regulate the *hrpXv* gene and the *hrpA* locus, and have a repressing effect on its own transcription. HrpXv is speculated to activate the loci *hrpB* to *hrpF* as a homodimer. PIP-boxes in the promoters of *hrpB*, *C*, *D*, and *hrpF* are indicated as square blocks. IM: inner membrane; OM: outer membrane; +: activation of transcription; –: repression of transcription.

compasses the loci *hrpA* to *hrpF* (Bonas et al. 1991) is regulated by at least two transcriptional activators. HrpXv, a regulatory protein of the AraC/XylS family, activates five loci, *hrpB* to *hrpF* (Wengelnik and Bonas 1996), four of which (*hrpB*, *hrpC*, *hrpD*, and *hrpF*) contain a conserved promoter element, the PIP box (Fenselau and Bonas 1995). We predict that HrpXv acts as a dimer because its putative DNA binding site, the PIP box, contains a direct repeat (Wengelnik and Bonas 1996). *hrpE* is the only locus in the cluster that shows a low level of expression in complex medium, but induction of expression in XVM2 still requires *hrpXv* (Wengelnik and Bonas 1996). Transcription of the *hrpXv* gene itself and that of *hrpA*, located at the left end of the large cluster, is independent of *hrpXv* but depends on *hrpG*. Hence, *hrpG* appears to be the key regulatory gene in the cascade activating *hrp* gene expression in *X. campestris* pv. *vesicatoria*. However, we can not exclude that an additional gene is involved in concert with *hrpG* and *hrpXv*. While *hrpXv* expression is not subjected to auto- or feedback regulation (Wengelnik and Bonas 1996), the HrpG protein seems to have a weak negative effect on transcription of the *hrpG* promoter (Fig. 5). The fact that *hrpG* expression is increased in XVM2 as compared with that in NYG, suggests that an as yet unknown activating component might be involved. Since HrpG shows homology to response regulator proteins of two-component systems and *hrp* gene activation depends on environmental stimuli like low concentration of phosphate and organic nitrogen, and presence of sucrose or fructose (Schulte and Bonas 1992b) we speculate that a corresponding 'sensor Hrp protein' exists and might be associated with the cytoplasmic membrane, like many sensor proteins in other systems (Parkinson and Kofoid 1992).

Although half of the genes in the large *hrp* clusters of *X. campestris* and *P. syringae* are conserved (Bogdanove et al. 1996), their mode of regulation seems to be fundamentally different. In *P. syringae*, *hrp* gene activation has been shown to depend on HrpR and HrpS. These proteins are 60% identical to each other and belong to the class of σ^{54} enhancer-binding proteins (Grimm et al. 1995; Grimm and Panopoulos 1989; Xiao et al. 1994). However, both HrpR and HrpS are significantly shorter than other proteins from this class and completely lack the N-terminal receiver domain. They activate transcription of *hrpL*, which encodes an alternate sigma factor and which recognizes a conserved sequence motif in the promoter regions of *hrp*- and *avr* loci (Xiao and Hutcheson 1994). It should be noted that although HrpR and HrpS are homologous to response regulators of two-component systems like XylR of *P. putida* (Morett and Segovia 1993) they do not share sequence similarity with HrpG of *X. campestris* pv. *vesicatoria*. In contrast to the proteins mentioned above, HrpG lacks a σ^{54}-interacting domain.

In no *hrp*-containing bacterium genes involved in sensing the environmental stimuli leading to *hrp* gene activation have been identified. In *X. campestris* pv. *vesicatoria*, isolation of a corresponding 'sensor' gene remains a prerequesite to consolidate our proposed model on *hrp* gene activation. Many two-component gene pairs from other species are located adjacent to one another or are even cotranscribed (Albright et al. 1989). From our analysis of the region downstream of *hrpG* it is very unlikely that the 'sensor' gene is located in close vicinity of *hrpG*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions.

The bacterial strains and plasmids used are described in Table 1. The genomic library of *X. campestris* pv. *vesicatoria* 75-3 was described previously (Minsavage et al. 1990). Plasmids were introduced into *Escherichia coli* by electroporation, and into *Xanthomonas* by conjugation using pRK2013 as helper plasmid in triparental matings (Ditta et al. 1980; Figurski and Helinski 1979). *E. coli* cells were cultivated at 37°C in Luria-Bertani medium and *Xanthomonas* strains at 28°C in NYG (Daniels et al. 1984), XVM2 (Wengelnik et al. 1996), or M9 (Ausubel et al. 1996). Ampicillin, cycloheximide, kanamycin, rifampicin, spectinomycin, and tetracycline were used at 100, 50, 25, 100, 100, and 10 μg per ml, respectively.

Table 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
Xanthomonas campestris pv. *vesicatoria*		
85-10	Pepper race 2; wild type; Rif^r	Bonas et al. 1989
85E	*gumG*-Tn*3-gus* insertion mutant of 85-10 *hrp*$^+$, EPS^-; Rif^r, Km^r	Wengelnik et al. 1996
85EX	*hrpXv* insertion mutant of 85E; Rif^r, Km^r, $Spec^r$	Wengelnik and Bonas 1996
85ΔAD	*hrpA1* to *hrpD4* deletion mutant of 85-10; Rif^r, $Spec^r$	This study
Escherichia coli		
DH5α	F^- *recA* φ80d*lacZ* Δ*M15*	BRL, Bethesda
Plasmids		
pLAFR3	RK2 replicon Mob^+ Tra^-; Tc^r; contains p*lac*	Staskawicz et al. 1987
pLAFR6	pLAFR3 derivative; multiple cloning site flanked by transcriptional terminators ; Tc^r	Bonas et al. 1989
pUC118	ColE1 replicon; Ap^r	Vieira and Messing 1987
pBluescript-KS II	Phagemid, pUC-derivative; Ap^r	Stratagene, La Jolla, CA
pL6GUSB	Promoterless *gusA* gene in pLAFR6; Tc^r	Knoop et al. 1991
pL3GUS	*gusA* gene under control of p*lac* in pLAFR3	B. J. Staskawicz
pXV74	pLAFR3 clone from *X. campestris* pv. *vesicatoria* 75-3 genomic library, containing *hrpA* to *hrpF*	Wengelnik et al. 1996
pXV9::A14, pXV9::B35, pXV9::C17, pXV9::D54	*hrp*-Tn*3-gus* insertion derivatives of pXV9	Schulte and Bonas 1992a
pXV4::E525	*hrpE*-Tn*3-gus* insertion derivative of pXV4	Wengelnik and Bonas 1996
pXV2::F312	*hrpF*-Tn*3-gus* insertion derivative of pXV2	Schulte and Bonas 1992b
pPX2	*hrpXv* promoter fragment in pL6GUSB	Wengelnik and Bonas 1996
pSX2	5-kb *Eco*RI-*Kpn*I fragment containing *hrpXv* in pLAFR6	Wengelnik and Bonas 1996
pRK2013	ColE1 replicon $TraRK^+$ Mob^+; Km^r	Figurski and Helinski 1979

Plant material and plant inoculations.

Inoculation of the near-isogenic pepper lines ECW and ECW-10R were performed as described (Bonas et al. 1991). To analyze bacterial growth in planta, suspensions of 10^5 bacteria per ml in 1 mM $MgCl_2$ were inoculated into leaves of the susceptible pepper cultivar ECW. Leaf disks were cut out at different time points after inoculation, macerated in 1 mM $MgCl_2$, and dilutions were plated on selective media.

Chemical-, transposon-, and marker exchange mutagenesis.

N-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) mutagenesis of *X. campestris* pv. *vesicatoria* 85E was performed essentially as described by Daniels et al. (1984), and subsequent mutant screening will be described elsewhere (Bonas et al., unpublished). Mutagenesis of cosmid pXV1 with transposon Tn*3-gus*, which carries a promoterless β-glucuronidase gene, was performed as described previously (Bonas et al. 1989). Transposon insertions were integrated into the genome of strain 85-10 by marker exchange (Bonas et al. 1991). The *hrpA* to *hrpD4* deletion mutant 85ΔAD was constructed as described in Wengelnik et al. (1996) except that 85-10 was used as parental strain. Correct genomic insertion in all mutants was verified by Southern analysis.

Construction of *hrpG* subclones.

For analysis of the minimal DNA region needed for *hrpG* function the 1,716-bp *Bam*HI-*Kpn*I, 1,330-bp *Xba*I-*Kpn*I, and 1,177-bp *Sal*I-*Kpn*I fragments of pBG1 were first subcloned into pUC118 (giving pUG72, pUG73, and pUG74, respectively). The inserts of all three plasmids were isolated as *Hin*dIII-*Kpn*I fragments and cloned into pL6GUSB, to yield plasmids pSG72, pSG73, and pSG74, respectively (see Fig. 1). Plasmid pLG75 contains a 2.5-kb insert covering the N-terminal portions of the *hrpG* and the *hrpXv* genes in pLAFR3, and was obtained by digesting pXV1 with *Eco*RI and religation of the DNA fragment containing the vector. Standard molecular techniques were used (Ausubel et al. 1996).

To analyze expression of the *hrpG* promoter region DNA fragments were fused to the promoterless *gusA* gene in pL6GUSB. Plasmid pPG1 carries the 1,330-bp *Bam*HI-*Eco*RI fragment. pPG2 resulted from deleting the internal *Sac*I fragments of plasmid pSG74 (an additional *Sac*I site is present in the polylinker of pL6GUSB between the *Kpn*I site and the *gusA* gene) and carries a 343-bp *Sal*I-*Sac*I fragment (see Fig. 1).

DNA sequencing and analysis.

The 6.5-kb *Hin*dIII fragment (using the *Hin*dIII site of the polylinker) at the extremity of cosmid pXV1 was cloned in both orientations into pBluescript KS II resulting in plasmids pBG1 and pBGI1. Deletion plasmids of pBG1 and pBGI1 were obtained by digesting the plasmids with several restriction enzymes and religation of the DNA fragments containing the vector. pBG1 and several deletion plasmids were sequenced using custom primers and the T3 and T7 primers with the T7 DNA polymerase sequencing kit (Pharmacia; Uppsala, Sweden), the Fidelity kit (Oncor, Gaithersburg, MD) or the ABI PRISM Dye Terminator Cycle Sequencing kit (Perkin Elmer, Foster, CA). Both strands of the 1,716-bp *Bam*HI-*Kpn*I fragment encompassing *hrpG* were sequenced, while only one strand of the 584-bp sequence downstream of the *Kpn*I site and further downstream regions was sequenced. The 1,559-bp sequence of *hrpG* from the *Eco*RV to the *Kpn*I site has been deposited in GenBank. The first six nucleotides are the reverse complement of the first six nucleotides in the published sequence of *hrpXv* (Wengelnik and Bonas 1996). Position 1 in Figure 3 corresponds to position 383 in the deposited sequence.

Tn*3-gus* insertion sites were sequenced using an oligonucleotide complementary to a sequence in the N-terminus of the β-glucuronidase gene (5' GATTTCACGGGTTGGGG 3').

Sequences were analyzed using the University of Wisconsin GCG 8.0 package (Devereux et al. 1984). Sequence comparisons were done with the BESTFIT program and the multiple sequence alignment in Figure 4 was created by PILEUP. Database searches were done using TBLAST (Altschul et al. 1990).

Determination of β-glucuronidase activities.

β-Glucuronidase (GUS) activities were determined after growth of *Xanthomonas* for 14 to 16 h in NYG or XVM2 by measurement of fluorescence using 4-methylumbelliferyl β-D-glucuronide as substrate as described previously (Schulte and Bonas 1992a; Wengelnik and Bonas 1996).

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