MPMI Vol. 8, No. 6, 1995, pp. 845–854. © 1995 The American Phytopathological Society

Sequence and Expression Analysis of the *hrpB* Pathogenicity Operon of *Xanthomonas campestris* pv. *vesicatoria* Which Encodes Eight Proteins with Similarity to Components of the Hrp, Ysc, Spa, and Fli Secretion Systems

Stefan Fenselau[1] and Ulla Bonas[2]

[1]Institut für Genbiologische Forschung Berlin GmbH, 14195 Berlin, Germany; [2]CNRS Institut des Sciences Végétales, Avenue de la Terrasse, 91198 Gif-sur-Yvette, France

Received 3 August 1995. Accepted 11 September 1995.

In this paper we describe the molecular characterization of *hrpB*, the largest operon in the *Xanthomonas campestris* pv. *vesicatoria hrp* cluster. The *hrpB* region encompasses 6 kb and encodes eight putative proteins, seven of which were expressed in *Escherichia coli*. The HrpB3 protein is the only one carrying a signal peptide sequence at the N-terminus and is a putative lipoprotein localized in the outer membrane of *X. campestris* pv. *vesicatoria*. The HrpB4 and HrpB8 proteins contain one and five putative transmembrane domains, respectively, and are most likely associated with the inner membrane. The HrpB3, HrpB5, HrpB6, and HrpB8 proteins show sequence similarity to putative components of different type III protein secretion pathways in bacteria. Examples include Hrp proteins from other plant pathogens, YscJ, YscN, YscL, and YscT of *Yersinia* spp., and MxiJ, Spa47, and Spa29 of *Shigella flexneri*. The transcription start site and the *hrpB* promoter was identified. The minimal *hrpB* promoter region of 90 bp contains a novel sequence motif, the PIP-box, which might play a role in transcription activation of the *hrpB* operon and possibly other plant-induced genes of *X. campestris* pv. *vesicatoria*.

Additional keywords: bacterial spot; flagellum; hypersensitive reaction; *Salmonella*.

Plant pathogenic bacteria have evolved a number of tools to overcome the host's physical barriers and defense responses against infection by microorganisms. The repertoire includes toxins, hydrolytic enzymes such as proteases, cellulases, pectinases (reviewed by Daniels et al. 1988; Collmer and Bauer 1994) and additional, as yet uncharacterized, virulence factors. A common feature of most plant pathogenic gram-negative bacteria are *hrp* genes which are absent from non-pathogenic bacteria and which were originally defined for *Pseudomonas syringae* pv. *phaseolicola* by Lindgren et al. (1986). Mutations in *hrp* genes lead to a pleiotropic phenotype, i. e., lack of induction of a hypersensitive reaction (HR) in resistant host and nonhost plants, and lack of disease development in susceptible host plants (reviewed by Willis et al. 1991; Bonas 1994).

Our research is focused on the pathogen *Xanthomonas campestris* pv. *vesicatoria*, the causal agent of bacterial spot disease of pepper and tomato plants. In susceptible plants, the bacteria colonize the intercellular spaces of the plant tissue and cause lesions which progress from a water-soaked state to a necrotic state. Infection of a resistant host plant results in the induction of a localized HR and cessation of bacterial multiplication (Minsavage et al. 1990; Brown et al. 1993). Plant resistance is specified by particular resistance genes in the host and corresponding avirulence genes in the bacterium and has mostly been described for the host plant pepper (Minsavage et al. 1990). The interactions between *X. campestris* pv. *vesicatoria* and both susceptible and resistant plants are controlled by *hrp* genes, which were identified by complementation of nonpathogenic strains carrying NTG-induced mutations. Genetic analysis using Tn*5* and Tn*3-gus* insertions has revealed that the *hrp* genes map to a 25-kb chromosomal region and are organized in at least six genetic loci, designated *hrpA* to *hrpF* (Bonas et al. 1991). Using Tn*3-gus* transposon insertions with β-glucuronidase as a reporter gene, expression of the *hrp* loci has been shown to be suppressed in complex medium but induced in planta and in vitro in "tomato conditioned medium" (TCM; Schulte and Bonas 1992).

One major goal of the molecular analysis of the *hrp* cluster is the elucidation of *hrp* gene function. A prerequisite for this is the analysis of its DNA sequence. The first sequence similarities, discovered between the putative HrpA1, HrpB3, HrpB6, and HrpC2 proteins of *X. campestris* pv. *vesicatoria* and proteins of mammalian bacterial pathogens gave a clue to what their biochemical function might be (Fenselau et al. 1992). The related proteins are involved in secretion of proteinaceous virulence factors in *Yersinia* spp. and *Shigella flexneri*, and of flagellum structural components in various bacteria (reviewed in Van Gijsegem et al. 1993).

Corresponding author: U. Bonas; E-mail: bonas@trefle.isv.cnrs-gif.fr

The sequence of the *hrpB* operon, together with the downstream *hrpA1* gene, can be retrieved from GenBank, accession #U33548.

Here we report the molecular characterization of the *hrpB* operon, which encompasses 6 kb and is flanked by *hrpA* and *hrpC*. The *hrpB* DNA sequence analysis predicts eight genes, seven of which could be expressed in *E. coli*. The promoter region of *hrpB* was defined by primer extension analysis and expression studies, and led to the identification of a novel sequence motif, the PIP-box.

RESULTS AND DISCUSSION

DNA sequence analysis of the *hrpB* operon.

The extent of the *hrpB* operon of *X. campestris* pv. *vesicatoria* was genetically defined by transposon Tn*3*-*gus* insertions #35 and #2, and Tn*5* insertions #14 and #77 (Bonas et al. 1991; Fig. 1). The orientation of *hrpB* transcription was deduced from GUS-active insertions within *hrpB* and found to be the same as the downstream *hrpA* locus but divergent from the upstream *hrpC* locus (Schulte and Bonas 1992). The transcriptional organization of *hrpB* was confirmed by complementing the Tn*3*-*gus* insertion mutant 85-10::*hrpB*35 using the 7.3-kb *Bam*HI fragment subcloned from pXV9 into pLAFR3 (Fig. 1). Plasmid pL373 in which expression of the genes is under the control of the *lacZ* promoter (constitutive in *X. campestris* pv. *vesicatoria*), but not pL337, was able to complement. The orientation-dependent complementation suggested that the 7.3-kb *Bam*HI fragment does not carry the *hrpB* promoter.

The DNA sequence of the 6-kb *hrpB* operon was determined. Eight ORFs were identified and found to have a codon usage typical for *Xanthomonas* genes. The genes were designated *hrpB1* to *hrpB8* (Fig. 1). The GTG codon at position 338 which is preceded by a putative ribosome binding site is most likely used as translation start of HrpB1 according to codon preference studies and primer extension data (Fig. 2; see below). The ATG of the second ORF starts 33 nucleotides after the TGA stop codon of *hrpB1*, and is also preceded by a putative ribosome binding site. Translation of this and the following six ORFs (encoding HrpB3–HrpB8) is probably coupled, i. e., the ATG codons either overlap with the last nucleotides or are present right after the stop codon of the previous ORF.

The insertion sites of transposon insertions in the operon have been sequenced. Tn*3*-*gus* insertions #35 and #2 interrupt translation after codon 99 in the *hrpB2* gene and after codon 75 in the *hrpB7* gene, respectively. Tn*5* insertions #14 and #77 are localized within *hrpB8* (K. Wengelnik and U. Bonas, unpublished). No typical transcription termination sequence is present downstream of the *hrpB8* gene which contains the promoter of the downstream *hrpA* locus. Transcription of *hrpA* starts 63 bp upstream of the *hrpB8* stop codon (K. Wengelnik and U. Bonas, unpublished).

Mapping of the *hrpB* transcription start site.

RNA dot blot experiments indicated that *hrpB* expression is induced 30 to 60 min after transfer of *X. campestris* pv. *vesicatoria* from NYG medium into TCM, and *hrpB* RNA is still detectable at 22 h after transfer (data not shown). To determine the transcription start of the *hrpB* operon, oligonucleo-

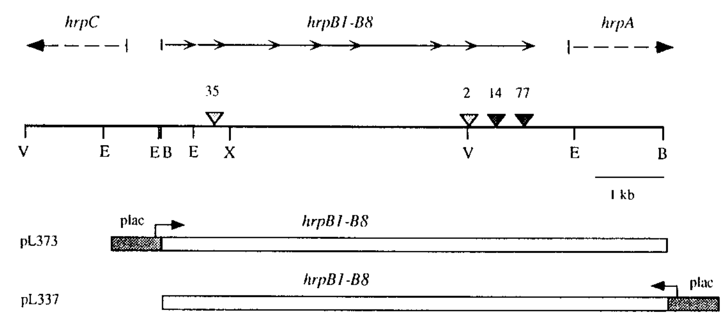

Fig. 1. Transcriptional organization of the *hrpB* region in *Xanthomonas campestris* pv. *vesicatoria*. The *hrpB* operon contains 8 genes, *hrpB1*-*hrpB8*, and is flanked upstream by *hrpC* and downstream by *hrpA*. The arrowheads indicate direction of transcription. Only relevant restriction sites are shown: B = *Bam*HI; E = *Eco*RI; V = *Eco*RV, X = *Xho*I. The triangles indicate the positions of sequenced transposon insertions mentioned in the text (#2, #35: Tn*3*-*gus*; #14, #77: Tn*5*). The plasmids pL373 and pL337 contain the 7.3-kb *Bam*HI fragment in different orientations with respect to the *lacZ* promoter in pLAFR3.

```
TAACGCGCCGAAGAGCACCGCCGCTGCAGTGACGTCCGGGCTGACCGGAACTTCCCCGTCCTTGCGCGCATCCCGCAGTTTCTTCTCGGTCGGCTTCTCG 100

                                                           PvuII
GTTTTTTCTTCCGACATTGCCTTCTCCTTCCGCGATCGCTGCGCCCCCGATTGTGGGCAGCTGTGGTGGAGCGATCCGTTTTCCAGGCGAATGTGTGGAT 200
                         ------ PIP-box ------            -35                      -10
                                 EcoRI.                   BamHI                                 +1
GGCCTGGCGAAGCTGGAATGATTTCTTCGCCAGCGAATTCCGATATTCGCTTCCGAAAGGATCCGGCCGGCGTGGCGAGCGTAGCGTTCCCCACGTGTAC 300

                          S/D      HrpB1
GGTCCGCATGCCATTAAAAACGAGGTGCAGTGTCGAGAAGACTCAATGTCCCGGGTCGGTCGTGAGTGGGCTCATCGAACTGATCACGGTCGGACTCACC 400

CATGAAAAGATTCAGGACGCTGCCGCTGTGCTGGCCGCAGTCCGCGTCCTGCGGCCAGAGCTGAAAGCGCTGGACA                        476
```

Fig. 2. Promoter region of the *hrpB* operon of *Xanthomonas campestris* pv. *vesicatoria*. The DNA sequence shown starts with the first nucleotide of oligonucleotide B3 and ends with the first nucleotide of pKS3 deletion clone pKSΔ2.1 which extends to the left. The transcription start site (+1) is marked with a dot at position 295; the putative ribosome binding site (S/D) is indicated. Putative −35/−10 elements and translation start site are underlined. The boxed sequences refer to the PIP-box. Only relevant restriction sites are shown.

tides were designed based on the sequence of the 5′-region of the putative *hrpB1* ORF (Fig. 2). Figure 3 shows a typical primer extension experiment. RNAs isolated from *X. campestris* pv. *vesicatoria* strain 85-10 after growth in different media were used for primer extension with oligonucleotide #58. *hrpB*-specific extension products were clearly detected after *hrp* gene induction in TCM (15 h) or after 3 days of growth in pepper ECW (susceptible line). Experiments using RNA isolated from bacteria grown in 2MS medium (Fig. 3) or in NYG medium (not shown) did not result in a complementary DNA. The transcription start site of *hrpB* is at position 295 (G; +1) in Figure 2, which was confirmed using oligonucleotides #59 and PEXB. This result suggests that the GTG codon located 37 nucleotides downstream, at position 332, is the translation start of HrpB1. The next GTG codon is 30 nucleotides further downstream but unlikely to be the start site since there is no ribosome binding site.

The PIP-box-containing *hrpB* promoter.

To identify the regulatory region needed for *hrpB* expression, several fragments were subcloned into promoter probe plasmid pL6GUSB, and their activity was measured after growth of *X. campestris* pv. *vesicatoria* under different conditions using *gusA* as a reporter gene (Fig. 4). The constructs pPB3, pPB4, and pPB5 that were shortened at the 5′-end, all carried the region up to +180 (with respect to the +1 transcription start) to facilitate subcloning. After transfer of the plasmids into *X. campestris* pv. *vesicatoria* strain 85-10, the transconjugants were grown for 14 h in NYG or in TCM, or for 3 days in the susceptible pepper plant ECW. While expression in all cases was suppressed in complex NYG medium (0.02 units/10^{10} CFU), induction occurred in TCM or in pepper (2 to 12 units/10^{10} CFU). These activities are in the same order of magnitude as obtained previously for transcriptional fusions in Tn*3-gus* insertion derivatives (Schulte and Bonas 1992). Fragments containing at least 90 bp upstream of the determined transcription start site, as in pPB1, pPB3 and pPB4, were found to be inducible in TCM and in the plant, and thus contain the entire *hrpB* promoter. Removal of a further 30 bp from the 5′-end completely abolished promoter activity (pPB5, starting from the *Eco*RI site). These results explain why the 7.3-kb *hrpB Bam*HI fragment, which contains 35 bp of upstream sequence, can only complement a *hrpB* mutant when expressed under control of the *lacZ* promoter in pLAFR3 (Figs. 1 and 2). Surprisingly, construct pPB2 which contains a 294-bp upstream region but ends right after the transcription start site is only weakly active (0.1 to 0.3 units/10^{10} CFU).

Inspection of the *hrpB* promoter sequence revealed only weak homology to the *E. coli* canonical sequence elements of sigma 70 transcription factor regulated promoters. There is no homology between the *hrpB* promoter and the consensus for sigma 54-dependent promoters. Little information is available on other promoter sequences in *Xanthomonas*. The consensus motif described for promoters of *hrp* genes and *hrp*-dependent *avr* genes in pathovars of *Pseudomonas syringae* (Salmeron and Staskawicz 1993; Innes et al. 1993; Xiao and Hutcheson 1994) is not present in the *hrpB* promoter of *X. campestris* pv. *vesicatoria*. Instead, a novel sequence motif, TTCGC-N15-TTCGC, is present 44 bp upstream of the *hrpB* transcription start site. This sequence is partially deleted in the inactive promoter construct pPB5 (Fig. 4). We have named this motif PIP-box (*P*lant *I*nducible *P*romoter). In summary, a fragment containing 90 bp upstream of the putative transcription start (pPB4) is sufficient for regulated promoter activity. This construct contains only 21 bp upstream of

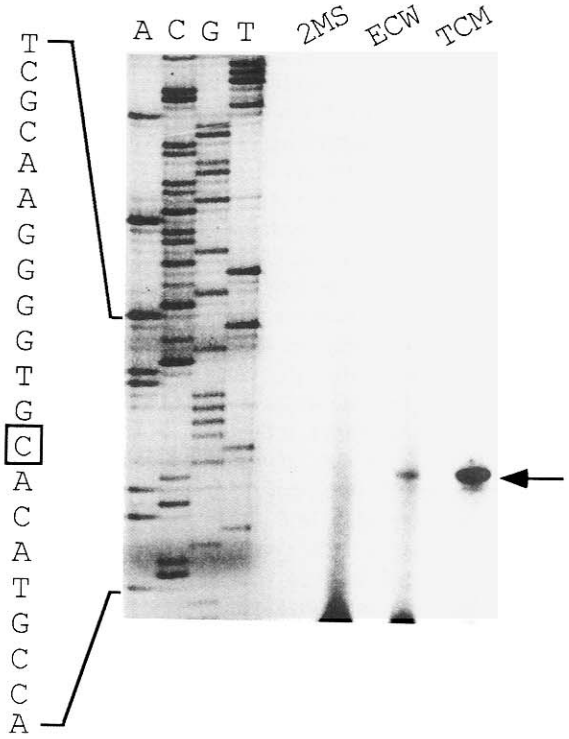

Fig. 3. Mapping of the *hrpB* transcription start site by primer extension analysis. The RNAs were extracted from strain 85-10 after growth in pepper ECW (3 d), TCM (15 h), and 2MS medium (15 h), annealed with oligonucleotide #58, and used as templates for reverse transcriptase. The sequence is the reverse complement of the coding strand, and was obtained with plasmid pKS3 as template and oligonucleotide #58 as primer.

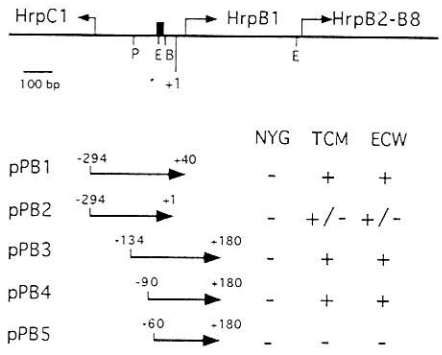

Fig. 4. Activity of *hrpB* promoter fragments in *Xanthomonas campestris* pv. *vesicatoria* strain 85-10. The top part illustrates the *hrpB/C* intergenic region. The positions of translation start sites of the first genes in the *hrpB* operon and of the divergent *hrpC1* are indicated by arrows; the black box refers to the PIP-box. Relevant restriction sites are indicated: *Bam*HI (B), *Eco*RI (E), and *Pvu*II (P). The positions of DNA fragments subcloned into promoter probe vector pL6GUSB are indicated in the lower part of the figure. The arrows refer to orientation of transcription. β-glucuronidase activity was measured in vitro (NYG or TCM medium) or in pepper line ECW (see Materials and Methods for details). (+) activity in the range of 2 to 12 units/10^{10} CFU; (–) no detectable activity, (+/–) weak activity (0.1 to 0.3 units/10^{10} CFU).

the PIP-box. The weak activity of construct pPB2 indicates that sequences downstream of the transcription start might be needed for optimal promoter activity or for RNA stability. Similar observations have been made in other bacterial systems, e. g., with the *flgF* promoter of *Caulobacter crescentus*. Marques and Gober (1995) attributed the effect of a downstream element to transcriptional activation of *flgF* rather than to RNA stability.

Identical PIP-boxes with the same spacing to the transcription start site are present also in the promoters of the *hrpC*, *hrpD,* and *hrpF* loci of *X. campestris* pv. *vesicatoria* (U. Bonas, unpublished), suggesting an important role of this motif in transcriptional control of *hrp* genes. Future experiments will show whether the *hrp* regulatory protein HrpX, the gene of which was recently isolated from *X. campestris* pv. *vesicatoria* (K. Wengelnik and U. Bonas, unpublished) binds to the PIP-box sequences thereby activating transcription. Interestingly, a PIP-box with the same spacing as in *hrpB* is also present in the putative promoter region of the avirulence gene *avrRxv* from *X. campestris* pv. *vesicatoria* (Whalen et al. 1993). This might indicate that the *avrRxv* gene is regulated in a similar way to *hrp* genes. If this is so, it would be in contrast to *avrBs3*, which was shown to be constitutively expressed (Knoop et al. 1991), but reminiscent of the situation in pathovars of *P. syringae*. In *P. syringae*, expression of several avirulence genes is under control of the regulatory genes *hrpRS* (Grimm et al. 1995) and *hrpL*, which regulate also *hrp* expression (Salmeron and Staskawicz 1993; Shen and Keen 1993; Innes et al. 1993; Xiao and Hutcheson 1994; Xiao et al. 1994).

Moreover, we found a similar motif (TTCGG-N15-TTCGC) upstream of the first gene in *hrp* transcription unit II of *Pseudomonas solanacearum* which is homologous to the *hrpB* operon of *X. campestris* pv. *vesicatoria*. PIP-boxes are also present upstream of *hrp* transcription units III and IV (Van Gijsegem et al. 1995) which are related to the *hrpC* and *hrpD* loci, respectively, of *X. campestris* pv. *vesicatoria* (U. Bonas, unpublished). Since transcription start sites have not been defined for any of the *P. solanacearum hrp* genes, the presence of PIP-boxes in front of genes that are regulated in a similar way as in *X. campestris* pv. *vesicatoria* is only an indication that this motif might also be important in other pathogenic bacteria.

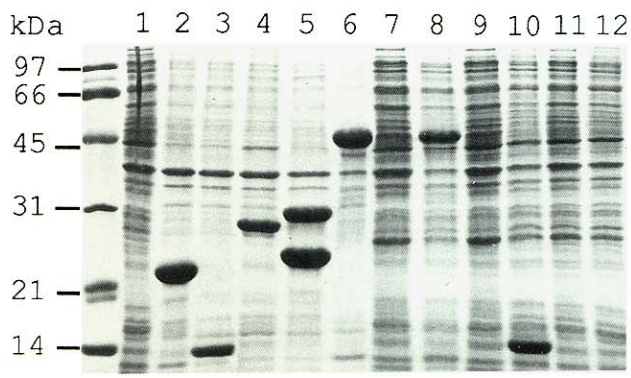

Fig. 5. Expression of *hrpB* sequences in *E. coli*. Proteins were separated on a 15% SDS polyacrylamide gel and stained with Coomassie Blue. Lanes next to the molecular weight marker contain: lane 1: total extract of induced E. coli BL21(DE3) (pET-3a). Lanes 2 to 6 show proteins of solubilized inclusion bodies of *E. coli* BL21(DE3) expressing *hrpB1* (lane 2), *hrpB2* (lane 3), *hrpB3* (lane 4), *hrpB4* + *hrpB5* (lane 5), and *hrpB6* (lane 6). Lanes 7 to 12 contain total extracts of *E. coli* BL21(DE3) pLysS carrying expression constructs of *hrpB6* (lane 7, 8), *hrpB7* (lane 9, 10; 16 kDa), and *hrpB8* (lane 11, 12), before (lanes 7, 9, 11) and after induction with IPTG (lanes 8, 10, 12).

Expression of *hrpB*-encoded proteins in *E. coli*.

To study expression of *hrpB* genes, DNA fragments carrying the different ORFs were ligated in frame into expression vector pET-3 which allows IPTG-inducible expression under the control of the T7 promoter in *E. coli*. To facilitate construction, some genes were cloned with small 5′ deletions (see Materials and Methods). This, and the fact that all proteins were translationally fused to the first 13 amino acids of the phage gene *10* protein should be considered for size estimation of the produced proteins which, except for HrpB7, were exclusively produced in inclusion bodies. As shown in Figure 5, the sizes of HrpB2, HrpB4, HrpB6, and HrpB7 were in good agreement with the predicted molecular mass of the proteins. Since the *hrpB4* construct also contained the *hrpB5* gene, expression of which is translationally coupled with HrpB4, two predominant proteins were observed (Fig. 5, lane 5). HrpB1, HrpB3, and HrpB5 had a lower mobility than expected. HrpB7 was detected only after transformation of the expression construct into *E. coli* carrying pLysS, which prevents expression of T7 directed genes under noninducing conditions (Studier et al. 1990). After induction with IPTG a protein of about 15 kDa was detected, which corresponds well to the expected size (Fig. 5, compare lanes 9 and 10). The HrpB8 protein was repeatedly undetectable even in *E. coli* carrying pLysS indicating that it might be toxic or poorly resolved by SDS-PAGE. This could be due to its five predicted membrane spanning domains. Because HrpB8 has significant sequence similarity to other (predicted) proteins, and Tn*5* insertions in *hrpB8* result in a mutant phenotype (Fig. 1), we believe that this protein is expressed in *X. campestris* pv. *vesicatoria*. HrpE, the HrpB8 counterpart of *P. solanacearum,* was visualized only in a coupled in vitro transcription-translation assay (Van Gijsegem et al. 1995).

In summary, seven HrpB proteins of *X. campestris* pv. *vesicatoria* could be expressed in *E. coli,* which is an important basis for further studies. The HrpA1 protein of *X. campestris* pv. *vesicatoria* is so far the only Hrp protein that was detected and localized in a plant pathogenic bacterium (K. Wengelnik and U. Bonas, unpublished).

Relevant features and sequence similarities of HrpB proteins.

The main characteristics of the eight predicted HrpB proteins and their sequence similarities are described below and summarized in Tables 1 and 2, which also gives the corresponding references.

HrpB1.

The HrpB1 protein is predicted to be 151 amino acids long, with an isoelectric point of 4.6. The protein contains several short hydrophobic regions which are, however, too short to span the membrane. The protein sequence is 36% identical and 63% similar to the 159 aa HrpK protein of *P. solanacearum*.

HrpB2.

The predicted HrpB2 protein (130 aa) is hydrophilic, has a high isoelectric point (9.4), and shares sequence similarity only to the HrpJ protein of *P. solanacearum*. The identity is 31%, and similarity 54%. The C-termini of both proteins are highly conserved: 14 out of the last 16 amino acids are identical and the remaining two are similar.

HrpB3.

As described earlier, the N-terminal region of HrpB3 resembles lipoprotein signal peptide sequences that are recognized by lipoprotein signal peptidase, with a putative cleavage site between amino acids 18 (alanine) and 19 (cysteine; position +1 of processed protein) (Fenselau et al. 1992). The serine residue in position +2 appears to determine the localization of the lipoprotein to the outer membrane (Braun and Wu 1994). The biosynthesis of lipoproteins involves post-translational modification of the invariant cysteine residue at the cleavage site by attachment of a diglyceride moiety which is subsequently fatty acylated (Wu and Tokunaga 1986). The amino acid sequence of the mature form of HrpB3 contains one hydrophobic domain near the C-terminus. Searches in the databank revealed that HrpB3 is most similar to several proteins from plant-associated bacteria: to HrpI of *P. solanacearum*, HrpC of *P. syringae*, and to NolT of *Rhizobium fredii*, which is a symbiont of soybean. Mutations in *nolT* cause a broader host spectrum of nodulation and a difference in protein secretion (Meinhardt et al. 1993). In addition, HrpB3 shares similarity to YscJ of *Yersinia* spp., to PrgK of *Salmonella typhimurium*, and to MxiJ of *Shigella flexneri*. These proteins play a role in secretion of proteinaceous virulence factors. All HrpB3 homologues are predicted to be lipoproteins. For YscJ in *Yersinia enterocolitica* (Michiels et al. 1991) and an MxiJ-PhoA fusion in *Shigella flexneri* (Allaoui et al. 1992), fatty acylation of the protein was demonstrated using [^{3}H]-palmitate. Attempts to visualize fatty acylated HrpB3 protein in *X. campestris* pv. *vesicatoria* were unsuccessful because of other labeled proteins in the same molecular weight range as HrpB3.

Table 1. Main features of predicted HrpB proteins from *Xanthomonas campestris* pv. *vesicatoria*

Protein	Length (aa)	Molecular mass (kDA)	Isoelectric point	Characteristics
HrpB1	151	16.0	4.6	Hydrophilic; no transmembrane domains
HrpB2	130	13.7	9.4	Hydrophilic
HrpB3 (+)	253	27.2	6.5	2 Hydrophobic domains; lipoprotein signal sequence
HrpB3	235	25.3	6.0	Processed form of HrpB3 (+); 1 hydrophobic domain
HrpB4	209	22.4	8.1	Hydrophilic
HrpB5	233	25.2	4.8	1 Small hydrophobic domain
HrpB6	442	47.7	4.9	Walker boxes A (P-loop), B, C (Mg^{2+}-binding motif)
HrpB7	169	18.8	7.6	Hydrophilic
HrpB8	276	29.0	4.7	5 Hydrophobic domains

Table 2. Protein sequence similarities between *Xanthomonas campestris* pv. *vesicatoria* HrpB proteins and other bacterial proteins; the values give %similarity/identity calculated using GAP

X. campestris pv. *vesicatoria*	HrpB1	HrpB2	HrpB3	HrpB4	HrpB5	HrpB6	HrpB7	HrpB8
Pseudomonas solanacearum	HrpK[a] 63/36%	HrpJ[a] 54/31%	HrpI[a] 70/59%	HrpH[a] 57/31%	HrpF[a] 52/33%	HrpE[a] 84/69%	HrpD[a] 42/24%	HrpC[a] 69/47%
Pseudomonas syringae			HrpC[b] 60/37%			HrpJ4[c] 66/47%		HrpX[d] 53/24%
Yersinia spp.			YscJ[e] 56/34%		LcrKc[f] 41/22%	YscN[g] 73/57%		YscT[h] 56/30%
Shigella flexneri			MxiJ[i] 52/27%			Spa47[j] 65/45%		Spa29[k] 53/26%
Salmonella typhimurium			PrgK[l] 56/28%			SpaL[m] 70/47%		SpaR[m] 54/26%
						FliI[n] 65/48%		
						InvC[o] 64/42%		
Bacillus subtilis						FlaA-ORF4[p] 68/50%		FliR[q] 52/24%
Escherichia coli						β-F1[r] 53/28%		FliR[s] 50/23%
Caulobacter crescentus								FliR[t] 50/26%
Erwinia carotovora								MopE[u] 50/23%
Rhizobium fredii			NolT[v] 61/41%					

References for the different proteins: [a]Van Gijsegem et al. 1995; [b]Preston et al. 1995; [c]Lidell and Hutcheson 1994; [d]Huang et al. 1995; [e]Michiels et al. 1991; [f]Rimpiläinen et al. 1992; [g]Woestyn et al. 1994; [h]Bergman et al. 1994; [i]Allaoui et al. 1992; [j]Venkatesan et al. 1992; [k]Sasakawa et al. 1993; [l]Pegues et al. 1995; [m]Groisman and Ochman 1993; [n]Vogler et al. 1991; [o]Eichelberg et al. 1994; [p]Albertini et al. 1991; [q]Carpenter et al. 1993; [r]Saraste et al. 1981; [s]Malakooti et al. 1994; [t]Zhuang and Shapiro 1995; [u]Mulholland et al. 1993; [v]Meinhardt et al. 1993.

HrpB4.

The HrpB4 protein is 209 aa in length. The sequence contains one putative transmembrane domain, from position 165 to 186, but no signal peptide sequence at the N-terminus according to the rules from Von Heijne (1986), suggesting that the protein is localized in the inner membrane. The only sequence similarity was found to the HrpH protein of *P. solanacearum* and extends over the entire lengths of the two proteins.

HrpB5.

The HrpB5 protein has an overall hydrophilic amino acid sequence with two small hydrophobic regions which are too short to span the membrane. The related HrpF protein of *P. solanacearum* is 70 amino acids longer than HrpB5; the C-terminal region of HrpF is remarkable in being highly acidic. Both HrpB5 and HrpF contain 22% alanine residues. The similarity of HrpB5 to the *Yersinia* LcrKc protein (= YscL) is weak but statistically significant as tested with the GCG program GAP in comparison to a randomized sequence. A sequence comparison of these three proteins is shown in Figure 6A.

HrpB6.

Among the proteins encoded by the *hrpB* operon, HrpB6 is the most conserved between species. Searches in the databank revealed previously that the HrpB6 protein is a putative ATPase (Fenselau et al. 1992). Since then, a number of homologues from plant and animal bacterial pathogens were found which all contain the Walker boxes A (P-loop) and B, a magnesium binding domain (Walker box C), and an αβ-signature (see Table 2). Interestingly, these putative ATPases share homology over the entire lengths of their protein sequences and are part of several different protein secretion systems translocating either factors involved in host or nonhost interactions, or flagellum structural proteins. Hence, the ATPase probably energizes the transport system. The ability to hydrolyze ATP has been recently demonstrated for the InvC protein of *S. typhimurium* (Eichelberg et al. 1994).

HrpB7.

HrpB7 (169 aa) is hydrophilic, and its sequence shares weak similarity with the HrpD protein of *P. solanacearum.*

HrpB8.

The HrpB8 protein (276 aa) contains five putative transmembrane domains. The lack of a signal peptide sequence at the N-terminus suggests that it is localized in the inner membrane. HrpB8 is related to numerous proteins, for example, to Spa29 and SpaR from *Shigella* and *Salmonella*, respectively, and to FliR proteins which play a role in flagellum assembly in different bacteria (see Fig. 6B). The HrpB8 counterparts also contain several putative transmembrane domains. To our knowledge, the localization of a HrpB8 homologue has in no case been demonstrated.

In summary, four HrpB proteins (HrpB3, HrpB5, HrpB6, HrpB8) are most similar to Hrp proteins in the plant pathogens *P. solanacearum* (Gough et al. 1992; Van Gijsegem et al. 1995) and *P. syringae* (Huang et al. 1995; Preston et al. 1995). The HrpB1, HrpB2, HrpB4, and HrpB7 proteins have only counterparts in *P. solanacearum* with sequence identities that are lower than for HrpB3, HrpB6, and HrpB8 and the corresponding homologues in *P. solanacearum* (see Table 2). Whether this points to a specialized function tailored for the particular bacterium-plant interaction has to await functional analysis. Interestingly, the genes in the *hrpB* operon of *X. campestris* pv. *vesicatoria* and the related genes present in *P. solanacearum* are organized colinearly and, except for *hrpB5*, are similar in length. Similarity between the two *hrp* regions of these taxonomically very different bacteria was already obvious from results of DNA hybridization experiments (Arlat et al. 1991) and suggests a common origin of the *hrp* genes in *X. campestris* pv. *vesicatoria* and *P. solanacearum.* The colinearity is, however, interrupted between the loci *hrpB* and *hrpA*, which is the locus downstream of the *hrpB8* gene, in *X. campestris* pv. *vesicatoria* (Fig. 1). In *P. solanacearum*, this region harbors the *hrpB* gene, which encodes the transcriptional activator of the *hrp* regulon (Genin et al. 1992). An equivalent gene is absent from the *hrp* cluster of *X. campestris* pv. *vesicatoria.*

To date, no sequence information on *hrpB* related genes from other xanthomonads is available. Recent studies on DNA amplification using specific primer pairs that correspond to sequences in the *hrpB5-B6-B7* region, and Southern hybridization suggest a high degree of sequence conservation among numerous pathogenic strains belonging to different pathovars of *X. campestris* or other *Xanthomonas* species (Leite et al. 1994a, 1994b). It was established that the amplification by PCR is sufficiently sensitive to differentiate pathogenic xanthomonads from nonpathogenic strains which normally lack the *hrp* cluster. Based on conservation on the amino acid level, discussed above, different primer combinations specific for the *hrpB* operon can now be designed and used for detection or phylogenetic analysis of pathogenic xanthomonads.

Among the new sequences reported here, the HrpB5 and HrpB8 proteins share similarities with type III secretion systems. This type of secretion system, which is distinct from the hemolysin (type I) and the *sec*-dependent (general or type II) systems was first described for secretion of Yop proteins by *Yersinia* spp. (Cornelis et al. 1989; Michiels et al. 1991). The Yop proteins lack a classical signal peptide sequence, are not processed, and require the presence of numerous Ysc and Lcr proteins for their polarized transport across both bacterial membranes (for a recent review see Forsberg et al. 1994). The finding of significant sequence conservation between bacterial Hrp proteins of plant pathogens and the putative components of transport systems for virulence factors of mammalian bacterial pathogens led to the hypothesis that *hrp* genes in plant pathogens encode a specialized protein secretion apparatus through which virulence proteins and elicitors of the plant defense response are secreted during infection (Fenselau et al. 1992; Gough et al. 1992). *hrp*-dependent secretion of HR-inducing proteins (harpins and PopA1) has indeed recently been shown for *Erwinia amylovora* (Wei and Beer 1993), *P. syringae* pv. *syringae* (He et al. 1993), and *P. solanacearum* (Arlat et al. 1994). For *X. campestris* pv. *vesicatoria*, the nature of secreted elicitors and virulence proteins is still enigmatic. Ultrastructural analysis of *hrp* mutants recently revealed that mutations in any of the six loci lead to localized deposition of papillae in the cell wall of pepper mesophyll cells which is suppressed in the interaction of the plant with a wild-type strain (Brown et al. 1995). The chemical nature of the suppressor(s) of the plant reaction is a matter of speculation, but it is conceivable that it could very well be a protein secreted in a *hrp*-dependent manner.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions.

The bacterial strains and plasmids used are described in Table 3. Plasmids were introduced into *Escherichia coli* by electroporation and into *Xanthomonas* by conjugation, using pRK2013 as a helper plasmid in triparental matings (Ditta et al. 1980; Figurski and Helinski 1979). *E. coli* cells were cultivated at 37°C in Luria-Bertani (LB) medium and *Xanthomonas* strains at 28°C in NYG broth (Daniels et al. 1984) or on NYG 1.5% agar. For in vitro studies of *hrp* gene expression bacteria were incubated in 2 MS (Murashige and Skoog medium containing 2% sucrose) or in tomato conditioned medium (TCM) as described previously (Schulte and Bonas 1992). Antibiotics were added to the media at the following final concentrations: ampicillin, 100 µg/ml; kanamycin, 50 µg/ml; tetracycline, 10 µg/ml; rifampicin, 100 µg/ml.

Plant material and plant inoculations.

Inoculation of the near-isogenic pepper lines ECW and ECW-10R, and reisolation of bacteria from plant tissue 3 days after inoculation were performed as described (Bonas et al. 1991).

Sequence analysis.

For sequence analysis fragments of pXV9 were subcloned into pBluescript KS generating pKE4.2, pKE10B, pKS3, and pKS4. A series of nested deletions of these plasmids were generated using DNaseI as previously described (Bonas et al. 1989). The DNA sequence of both strands of overlapping deletion subclones was determined by using commercial (Stratagene) or custom primers, and the T7 DNA polymerase sequencing kit containing deaza-dGTP (Pharmacia; Uppsala, Sweden). Tn*3-gus* insertion sites and promoter-*gusA* fusions were sequenced using an oligonucleotide complementary to the 5′-region of the β-glucuronidase gene (5′ GATTTCACGGGTTGGGG 3′). Sequence data were analyzed using the University of Wisconsin GCG package (Version 8.0; Devereux et al. 1984), PRODOM (Sonnhammer and Kahn 1994) and TBLAST (Altschul et al. 1990). GAP was used for sequence alignments applying the following parameters: gap weight of 3.0 and length weight of 0.1. Randomization of 10

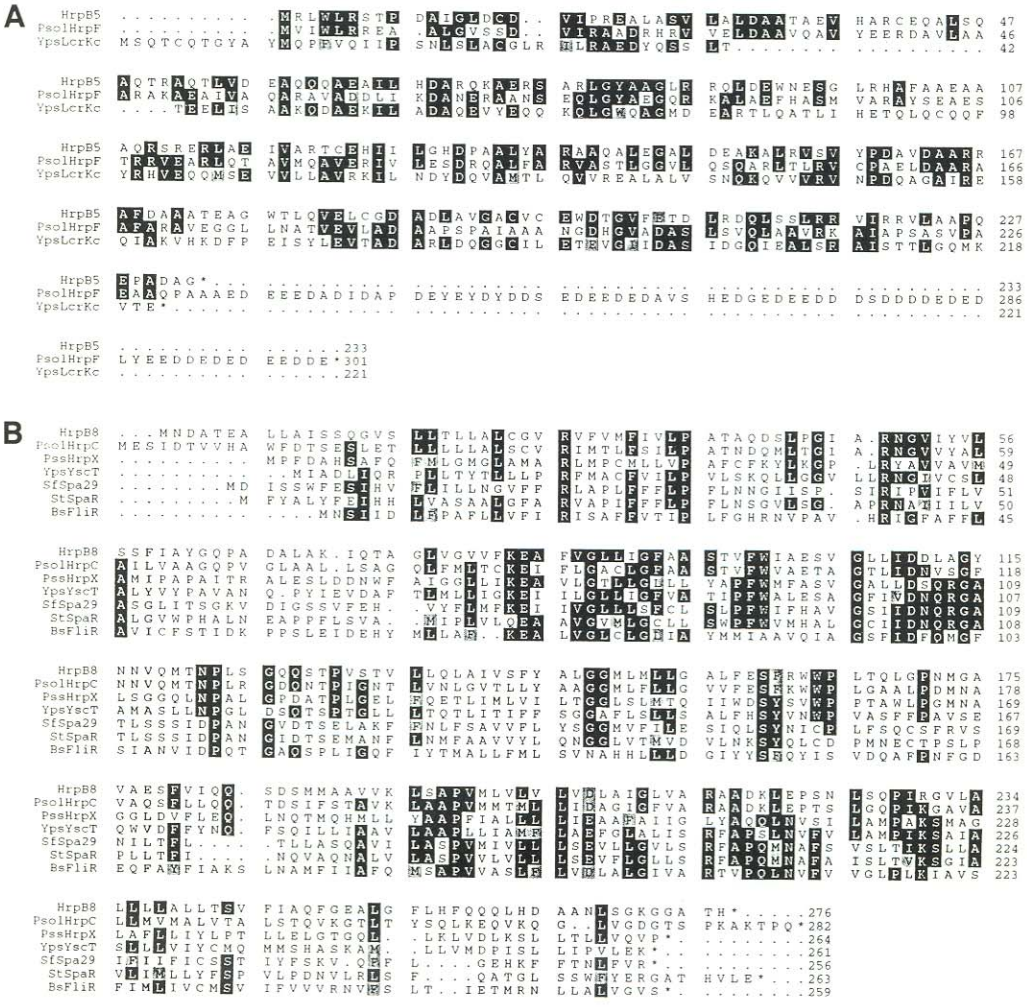

Fig. 6. Amino acid sequence alignments of HrpB5 and HrpB8 and their corresponding related proteins described in this paper. **A,** HrpB5 of *Xanthomonas campestris* pv. *vesicatoria*; HrpF of *P. solanacearum* (Van Gijsegem et al. 1995); and LcrKc of *Y. pseudotuberculosis* (Rimpiläinen et al. 1992). **B,** HrpB8 of *X. campestris* pv. *vesicatoria*; HrpC of *P. solanacearum* (Van Gijsegem et al. 1995); HrpX of *P. solanacearum* pv. *syringae* (Huang et al. 1995); YscT of *Y. pseudotuberculosis* (Bergman et al. 1994); Spa29 of *S. flexneri* (Sasakawa et al. 1993), SpaR of *S. typhimurium* (Groisman and Ochman 1993), and FliR of *B. subtilis* (Carpenter et al. 1993). The alignments were performed using PrettyPlot and Pileup.

sequences determined the average quality of alignments. The sequence of the *hrpB* operon, together with the downstream *hrpA1* gene, can be retrieved from GenBank, accession #U33548.

Primer extension.

Isolation of bacterial RNA (Aiba et al. 1981) and primer extension analysis (Ausubel et al. 1992) were performed essentially as described. The following oligonucleotides were used:

#58: (5′-CACTCACGACCGACCCGGGACATTGAGTC-3′);

#59: (5'-CAGCTCTGGCCGCAGGACGCGGACTGCG-3');

and PEXB (5'-TGAATCTTTTCATGGGTGAGTCCG-ACCGTG-3').

Fifty micrograms of bacterial RNA and a [^{32}P]-labeled oligonucleotide were annealed overnight at 37°C. Primer extension was performed with 200 units of Superscript II reverse transcriptase (Gibco-BRL) at 42°C for 60 min and the resulting extension products were analyzed on 6% denaturing polyacrylamide gels.

Construction of promoter subclones.

Standard molecular biological techniques were used (Sambrook et al. 1989). DNA fragments generated using PCR were subcloned into the *Hin*dIII–*Sac*I sites of pL6GUSB. The sequences of the oligonucleotides which contained a *Hin*dIII or *Sac*I site (italics) at their 5′-end, were as follows:

B1: 5'-TCGAT*AAGCTT*GGCGAAGCTGGAATGATTTG-3';

B3: 5'-TCGAT*AAGCTT*AACGCGCCGAAGAGCACCGCC-3';

B4: 5'-GCGGTG*GAGCTC*GTGGGGAACGCTACGCTCGC-3';

B5: 5'-GCGGTG*GAGCTC*ACACTGCACCTCGTTTTTAATG-3'.

For most constructs, pKSΔ2.1, a deletion clone of plasmid pKS3 was used; the first 476 bp of its insert sequence is depicted as reverse complement in Figure 2. Plasmid pPB1 contains a fragment amplified by PCR using oligonucleotides B3 and B5 which corresponds to positions 1 to 337 in the sequence shown in Figure 2. pPB2 was generated using primers B3 and B4 (position 1–295). The 320-bp *Pvu*II-*Sac*I fragment of clone pKSΔ2.1 is present in construct pPB3 (position 158–476). For pPB4 a 280-bp fragment was amplified from pKSΔ2.1 using oligonucleotide B1 and the T7 promoter primer (position 205–476), followed by *Hin*dIII-*Sac*I digestion. The insert of pPB5 is an *Eco*RI-*Sac*I fragment from pKSΔ2.1 (position 235–476) which was ligated into the *Bam*HI–*Sac*I site of pL6GUSB. The *Sac*I site of the latter constructs originates from pBluescript KS. The 3′ fusion sites of all constructs were sequenced using an oligonucleotide complementary to the 5′-region of the β-glucuronidase gene (see above). After transfer of the plasmids into *X. campestris* pv. *vesicatoria* strain 85–10, β-glucuronidase (GUS) activities were determined as described (Schulte and Bonas 1992).

Protein expression.

For *hrpB* expression in *E. coli* appropriate DNA fragments were subcloned into the filled-in *Bam*HI site of expression vectors pET-3a, 3b, or 3c (Studier et al. 1990) to generate translational fusions with the first 13 amino acids of the phage T7 gene *10* protein which results in an additional mass of the expressed protein of 1.4 kDa. For practical reasons several subcloned ORFs lacked N-terminal sequences. For *hrpB1* expression a 1-kb *Bam*HI–*Pvu*II fragment from pKS4 deletion clone #11-8 was ligated into pET-3c; this way the first 12 bp of the ORF are deleted. For *hrpB2*, a 445-bp *Spe*I (vector site)–*Pvu*II fragment from pKE4.2 deletion clone #2 was ligated into pET-3b; the first 17 bp of the gene are not present. The *hrpB3* gene was expressed in pET-3c from a 745 bp *Spe*I (vector site)–*Pvu*II fragment from pKE10B deletion clone #G25; to exclude possible problems due to the signal sequence the first 66 bp of the ORF were deleted. To express *hrpB4* and *hrpB5*, a 1,235-bp *Spe*I (vector site)–*Pvu*II fragment from pKE-4.2 deletion clone #6 was ligated into pET-

Table 3. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference/ source
X. campestris pv. *vesicatoria*		
85-10	Pepper race 2; wild type; Rif^R	Bonas et al. 1989
E. coli		
DH5α	F$^-$ *rec*A ϕ80*dlacZ* Δ*M15*	Bethesda Research Laboratories; Bethesda, MD
HMS174	F$^-$ *hsd*R *rec*A Rif^R	Studier and Moffat 1986
BL21(DE3)	F$^-$ *hsd*R *gal*; lysogenic for phage DE3; carries *lac*UV5-T7 gene *1*	Studier and Moffat 1986
Plasmids		
pBluescript II KS	phagemid, pUC-derivative; Ap^R	Stratagene; La Jolla, CA, USA
pET-3a, b, c	pT7 protein expression vectors; Ap^R	Studier et al. 1990
pLAFR3	RK2 replicon Mob$^+$Tra$^-$;Tc^R; contains p*lac*	Staskawicz et al. 1987
pLAFR6	RK2 replicon Mob$^+$Tra$^-$;Tc^R multicloning site flanked by transcriptional terminators	B. Staskawicz
pL6GUSB	Carries promoterless *gusA* gene in pLAFR6	B. Staskawicz
pXV9	pLAFR3 *hrpA-E* clone from *X. campestris* pv. *vesicatoria* 75-3	Bonas et al. 1991
pXV9::35	Tn*3-gus* insertion derivative of pXV9	Bonas et al. 1991
pXV9::2	Tn*3-gus* insertion derivative of pXV9	Bonas et al. 1991
pL373	7.3 kb *Bam*HI fragment from pXV9 in pLAFR3; expresses *hrpB* genes under control of p*lacZ*	This study
pL337	As pL373 but inverted orientation	This study
pKE4.2	4.3 kb *Eco*RV-*Eco*RI fragment of pXV9 in pBluescript II KS; contains internal part of *hrpB*	This study
pKE10B	5.5 kb *Eco*RI fragment of pXV9 in pBluescript II KS; contains *hrpA/B* region but inverted as compared to pKE4.2	This study
pKS3	3 kb *Xho*I-*Eco*RV fragment of pXV9 in pBluescript II KS; contains *hrpB/C* region	This study
pKS4	As pKS3; inverted orientation	This study

3c. This would be predicted to express both proteins full size. For *hrpB6* expression a 1.4-kb *Spe*I (vector site)–*Eco*RV fragment from pKE4.2 deletion clone #12a was ligated into pET-3b; the first 125 bp of the ORF are not present. For *hrpB7* and *hrpB8* expression a 425-bp *Stu*I–*Spe*I fragment (the first 145 bp are missing) and a 800-bp *Hin*dIII–*Spe*I fragment (the first 56 bp of the ORF are missing) from pKE10B deletion clones #G6c and #G2, respectively, were subcloned in pET-3b and 3a. The recombinant plasmids were transformed into *E. coli* strain HMS174 (Studier and Moffat 1986) and fusion sites of all constructs were sequenced.

For expression of the different *hrpB* genes, the plasmids described above were transformed into *E. coli* strain BL21 (DE3) with or without pLysS. The transformants were used for protein production by induction of an early log phase culture at 30°C for 1 h by IPTG (isopropyl-β-thiogalactopyranoside; 0.5 mM) to express the T7 RNA polymerase gene. After induction the cells were harvested by centrifugation, and proteins were extracted and analyzed by SDS-PAGE as described (Knoop et al. 1991). Briefly, total proteins were isolated by three passages of bacterial cell suspension through a French pressure cell. After centrifugation of the extract, aliquots of the supernatant or of the pellet (if containing inclusion bodies) were separated on 15% SDS-polyacrylamide gels in Laemmli buffer and visualized by Coomassie brilliant blue (Bio-Rad).

ACKNOWLEDGMENTS

We are extremely grateful to I. Balbo for excellent technical assistance, to F. Söling for contributing to the sequence analysis, to C. Boucher for communicating sequences before publication, and to G. Van den Ackerveken and K. Wengelnik for helpful comments on the manuscript. This research was supported in part by a grant from the EEC (BIOT-CT90-0168) to U. B.

LITERATURE CITED

Aiba, H., Adhya, S., and de Crombugghe, B. 1981. Evidence of two functional *gal* promoters in intact *Escherichia coli* cells. J. Biol. Chem. 256:11905-11910.

Albertini, A. M., Caramori, T., Crabb, W. D., Scoffone, F., and Galizzi, A. 1991. The *flaA* locus of *Bacillus subtilis* is part of a large operon coding for flagellae structures, motility functions, and an ATPase-like polypeptide. J. Bacteriol. 173:3573-3579.

Allaoui, A., Sansonetti, P. J., and Parsot, C. 1992. MxiJ, a lipoprotein involved in secretion of *Shigella* Ipa invasins, is homologous to YscJ, a secretion factor of the *Yersinia* Yop proteins. J. Bacteriol. 174:7661-7669.

Altschul, S. F., Gish, W., Miller, W., Meyers, E., and Lipman, D. J. 1990. Basic local alignment search tool. J. Mol. Biol. 215:403-410.

Arlat, M., Gough, C. L., Barber, C. E., Boucher, C., and Daniels, M. J. 1991. *Xanthomonas campestris* contains a cluster of *hrp* genes related to the larger *hrp* cluster of *Pseudomonas solanacearum*. Mol. Plant-Microbe Interact. 4:593-601.

Arlat, M., Van Gijsegem, F., Huet, J. C., Pernollet, J. C., and Boucher, C. A. 1994. PopA1, a protein which induces a hypersensitive-like response on specific *Petunia* genotypes, is secreted via the Hrp pathway of *Pseudomonas solanacearum*. EMBO J. 13:543-553.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., eds. 1992. Current Protocols in Molecular Biology. John Wiley & Sons, Inc., New York.

Bergman, T., Erickson, K., Galyov, E., Persson, C., and Wolf-Watz, H. 1994. The *lcrB* (*yscN/U*) gene cluster of *Yersinia pseudotuberculosis* is involved in Yop secretion and shows high homology to the *spa* gene clusters of *Shigella flexneri* and *Salmonella typhimurium*. J. Bacteriol. 176:2619-2626.

Bonas, U. 1994. *hrp* genes of phytopathogenic bacteria. Curr. Top. Microbiol. Immunol. 192:79-98.

Bonas, U., Stall, R. E., and Staskawicz, B. J. 1989. Genetic and structural characterization of the avirulence gene *avrBs3* from *Xanthomonas campestris* pv. *vesicatoria*. Mol. Gen. Genet. 218:127-136.

Bonas, U., Schulte, R., Fenselau, S., Minsavage, G. V., Staskawicz, B. J., and Stall, R. E. 1991. Isolation of a gene cluster from *Xanthomonas campestris* pv. *vesicatoria* that determines pathogenicity and the hypersensitive response on pepper and tomato. Mol. Plant-Microbe Interact. 4:81-88.

Braun, V., and Wu, H. C. 1994. Lipoproteins, structure, function, biosynthesis and model for protein export. Pages 319-341 in: J.-M. Ghuysen and R. Hakenbeck, eds. Bacterial Cell Wall. Elsevier Science B.V., Amsterdam.

Brown, I., Mansfield, J., Irlam, I., Conrads-Strauch, J., and Bonas, U. 1993. Ultrastructure of interactions between *Xanthomonas campestris* pv. *vesicatoria* and pepper, including immunocytochemical localization of extracellular polysaccharides and the AvrBs3 protein. Mol. Plant-Microbe Interact. 6:376-386.

Brown, I., Mansfield, J., and Bonas, U. 1995. *hrp* genes in *Xanthomonas campestris* pv. *vesicatoria* determine ability to suppress papillae deposition in pepper mesophyll cells. Mol. Plant-Microbe Interact. 8:825-836.

Carpenter, P. B., Zuberi, A. R., and Ordal, G. W. 1993. *Bacillus subtilis* flagellar proteins FliP, FliQ, FliR and FlhB are related to *Shigella flexneri* virulence factors. Gene 137:243-245.

Collmer, A., and Bauer, D. W. 1994. *Erwinia chrysanthemi* and *Pseudomonas syringae*: Plant pathogens trafficking in extracellular virulence proteins. Curr. Top. Microbiol. Immunol. 192:43-78.

Cornelis, G. R., Biot, T., Lambert de Rouvroit, C., Michiels, T., Mulder, B., Sluiters, C., Sory, M.-P., Van Bouchaute, M., and Vanooteghem, J.-C. 1989. The *Yersinia* yop regulon. Mol. Microbiol. 3:1455-1469.

Daniels, M. J., Barber, C. E., Turner, P. C., Sawczyc, M. K., Byrde, R. J. W. and Fielding, A. H. 1984. Cloning of genes involved in pathogenicity of *Xanthomonas campestris* pv. *campestris* using the broad host range cosmid pLAFR1. EMBO J. 3:3323-3328.

Daniels, M. J., Dow, J. M., and Osbourn, A. E. 1988. Molecular genetics of pathogenicity in phytopathogenic bacteria. Annu. Rev. Phytopathol. 26:285-312.

Devereux, J., Haeberli, P., and Smithson, O. 1984. A comprehensive set of sequence analysis programmes for the VAX. Nucleic Acids Res. 12:387-395.

Ditta, G., Stanfield, S., Corbin, D., and Helinski, D. 1980. Broad host range DNA cloning system for gram-negative bacteria: Construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA 77:7347-7351.

Eichelberg, K., Ginocchio, C. C., and Galan, J. E. 1994. Molecular and functional characterization of the *Salmonella typhimurium* invasion genes *invB* and *invC*: homology of InvC to the F_0F_1 ATPase family of proteins. J. Bacteriol. 176:4501-4510.

Fenselau, S., Balbo, I., and Bonas, U. 1992. Determinants of pathogenicity in *Xanthomonas campestris* pv. *vesicatoria* are related to proteins involved in secretion in bacterial pathogens of animals. Mol. Plant-Microbe Interact. 5:390-396.

Figurski, D., and Helinski, D. R. 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc. Natl. Acad. Sci. USA 76:1648-1652.

Forsberg, A., Rosquist, R., and Wolf-Watz, H. 1994. Regulation and polarized transfer of the *Yersinia* outer proteins (Yops) involved in antiphagocytosis. Trends Microbiol. 2:14-19.

Genin, S., Gough, C. L., Zischek, C., and Boucher, C. A. 1992. The *hrpB* gene encodes a positive regulator of pathogenicity genes from *Pseudomonas solanacearum*. Mol. Microbiol. 6:3065-3076.

Gough, C. L., Genin, S., Zischek, C., and Boucher, C. A. 1992. *hrp* genes of *Pseudomonas solanacearum* are homologous to pathogenicity determinants of animal pathogenic bacteria and are conserved among plant pathogenic bacteria. Mol. Plant-Microbe Interact. 5:384-389.

Grimm, C., Aufsatz, W., and Panopoulos, N. J. 1995. The *hrpRS* locus of *Pseudomonas syringae* pv. *phaseolicola* constitutes a complex regulatory unit. Mol. Microbiol. 15:155-165.

Groisman, E. A., and Ochman, H. 1993. Cognate gene clusters govern invasion of host epithelial cells by *Salmonella typhimurium* and *Shigella flexneri*. EMBO J. 12:3779-3787.

He, S. Y., Huang, H. C., and Collmer, A. 1993. *Pseudomonas syringae* pv. *syringae* $Harpin_{Pss}$: A protein that is secreted via the Hrp pathway and elicits the hypersensitive response in plants. Cell 73:1255-1266.

Huang, H.-C., Lin, R.-H., Chang, C.-J., Collmer, A., and Deng, W.-L. 1995. The complete *hrp* gene cluster of *Pseudomonas syringae* pv. *syringae* 61 includes two blocks of genes required for harpin$_{Pss}$ secretion that are arranged colinearly with *Yersinia ysc* homologs. Mol. Plant-Microbe Interact. 8:733-746.

Innes, R. W., Bent, A. F., Kunkel, B. N., Bisgrove, S. R., and Staskawicz, B. J. 1993. Molecular analysis of avirulence gene *avrRpt2* and identification of a putative regulatory sequence common to all known *Pseudomonas syringae* avirulence genes. J. Bacteriol. 175:4859-4869.

Knoop, V., Staskawicz, B. J., and Bonas, U. 1991. The expression of the avirulence gene *avrBs3* from *Xanthomonas campestris* pv. *vesicatoria* is not under the control of *hrp* genes and is independent of plant factors. J. Bacteriol. 173:7142-7150.

Leite, R. P., Minsavage, G. V., Bonas, U., and Stall, R. E. 1994a. Detection and identification of plant pathogenic strains of *Xanthomonas* based on amplification of DNA sequences related to the *hrp* genes of *Xanthomonas campestris* pv. vesicatoria. Appl. Environ. Microbiol. 60:1068-1077.

Leite, R. P., Egel, D. S., and Stall, R. E. 1994b. Genetic analysis of *hrp*-related DNA sequences of *Xanthomonas campestris* strains causing diseases of citrus. Appl. Environ. Microbiol. 60:1078-1086.

Lidell, M. C., and Hutcheson, S. W. 1994. Characterization of the *hrpJ* and *hrpU* operons of *Pseudomonas syringae* pv. *syringae* Pss61: Similarity with components of enteric bacteria involved in flagellar biogenesis and demonstration of their role in harpin$_{Pss}$ secretion. Mol. Plant-Microbe Interact. 7:488-497.

Lindgren, P. B., Peet, R. C., and Panopoulos, N. J. H. 1986. Gene cluster of *Pseudomonas syringae* pv. *phaseolicola* controls pathogenicity on bean plants and hypersensitivity on nonhost plants. J. Bacteriol. 168:512-522.

Malakooti, J., Ely, B., and Matsumura, P. 1994. Molecular characterization, nucleotide sequence, and expression of the *fliO*, *fliP*, *fliQ*, and *fliR* genes of *Escherichia coli*. J. Bacteriol. 176:189-197.

Marques, M. V., and Gober, J. W. 1995. Activation of a temporally regulated *Caulobacter* promoter by upstream and downstream sequence elements. Mol. Microbiol. 16:279-289.

Meinhardt, L.W., Krishnan, H. B., Balatti, P. A., and Pueppke, S. G. 1993. Molecular cloning and characterization of a *sym* plasmid locus that regulates cultivar-specific nodulation of soybean by *Rhizobium fredii* USDA257. Mol. Microbiol. 8:17-29.

Michiels, T., Vanooteghem, J.-C., Lambert de Rouvroit, C., China, B., Gustin, A., Boudry, P., and Cornelis, G. R. 1991. Analysis of *virC*, an operon involved in the secretion of Yop proteins by *Yersinia enterocolitica*. J. Bacteriol. 173:4994-5009.

Minsavage, G. V., Dahlbeck, D., Whalen, M. C., Kearney, B., Bonas, U., Staskawicz, B. J., and Stall, R. E. 1990. Gene-for-gene relationships specifying disease resistance in *Xanthomonas campestris* pv. *vesicatoria*-pepper interactions. Mol. Plant-Microbe Interact. 3:41-47.

Mulholland, V., Hinton, J. C. D., Sidebotham, J., Toth, I. K., Hyman, L. J., Pérombelon, M. C. M., Reeves, P. J., and Salmond, G. P. C. 1993. A pleiotropic reduced virulence (Rvi$^-$) mutant of *Erwinia carotovora* subspecies *atroseptica* is defective in flagella assembly proteins that are conserved in plant and animal bacterial pathogens. Mol. Microbiol. 9:343-356.

Pegues, D. A., Hantman, M. J., Behlau, I., and Miller, S. I. 1995. PhoP/PhoQ transcriptional repression of *Salmonella typhimurium* invasion genes: Evidence for a role in protein secretion. Mol. Microbiol. 17:169-181.

Preston, G. M., Huang, H.-C., He, S. Y., and Collmer, A. 1995. The HrpZ proteins of *Pseudomonas syringae* pvs. *syringae*, *glycinea* and *tomato* are encoded by an operon containing *Yersinia ysc* homologs and elicit the hypersensitive response in tomato but not soybean. Mol. Plant Microbe Interact. 8:717-732.

Rimpiläinen, M., Forsberg, A., and Wolf-Watz, H. 1992. A novel protein, LcrQ, involved in the low-calcium response of *Yersinia pseudotuberculosis* shows extensive homology to YopH. J. Bacteriol. 174:3355-3363.

Salmeron, J. M., and Staskawicz, B. J. 1993. Molecular characterization and *hrp* dependence of the avirulence gene *avrPto* from *Pseudomonas syringae* pv. tomato. Mol. Gen. Genet. 239:6-16.

Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular Cloning. A Laboratory Manual, 2nd ed. Cold Spring Harbor Press, Cold Spring Harbor, N. Y.

Saraste, M., Gay, N. J., Eberle, A., Runswick, M. J., and Walker, J. E. 1981. The *atp* operon: Nucleotide sequences of the genes for the α, β, and γ subunits of *Escherichia coli* ATP synthase. Nucleic Acids Res. 9:5287-5296.

Sasakawa, C., Komatsu, K., Tobe, T., Fukuda, I., Suzuki, T., and Yoshikawa, M. 1993. Eight genes in region 5 that form an operon are essential for invasion of epithelial cells by *Shigella flexneri*. J. Bacteriol. 175:2334-2346.

Schulte, R., and Bonas, U. 1992. The expression of the *hrp* gene cluster from *Xanthomonas campestris* pv. *vesicatoria* that determines pathogenicity and hypersensitivity on pepper and tomato, is plant-inducible. J. Bacteriol. 174:815-823.

Shen, H., and Keen, N. T. 1993. Characterization of the promoter of avirulence gene D from *Pseudomonas syringae* pv. tomato. J. Bacteriol. 175:5916-5924.

Sonnhammer, E. L. L., and Kahn, D. 1994. Modular arrangement of proteins as inferred from analysis of homology. Protein Sci. 3:482-492.

Staskawicz, B. J., Dahlbeck, D., Keen, N., and Napoli, C. 1987. Molecular characterization of cloned avirulence genes from race 0 and race 1 of *Pseudomonas syringae* pv. *glycinea*. J. Bacteriol. 169:5789-5794.

Studier, F. W., and Moffatt, B. A. 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J. Mol. Biol. 189:113-130.

Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. 1990. Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol. 185:60-89.

Van Gijsegem, F., Genin, S., and Boucher, C. A. 1993. Conservation of secretion pathways for pathogenicity determinants of plant and animal bacteria. Trends Microbiol. 1:175-180.

Van Gijsegem, F., Gough, C., Zischek, C., Niqueux, E., Arlat, M., Genin, S., Barberis, P., German, S., Castello, P., and Boucher, C. A.. 1995. The *hrp* gene locus of *Pseudomonas solanacearum* that controls the production of a type III secretion system, encodes eight proteins related to components of the bacterial flagellar biogenesis complex. Mol. Microbiol. 15:1095-1114.

Venkatesan, M. M., Buysse, J. M., and Oaks, E. W. 1992. Surface presentation of *Shigella flexneri* invasion plasmid antigens requires the products of the *spa* locus. J. Bacteriol. 174:1990-2001.

Vogler, A. P., Homma, M., Irikura, V. M., and Macnab, R. M. 1991. *Salmonella typhimurium* mutants defective in flagellar filament regrowth and sequence similarity of FliI to F_0F_1, vacuolar, and archaebacterial ATPase subunits. J. Bacteriol. 173:3564-3572.

Von Heijne, G. 1986. A new method for predicting signal sequence cleavage sites. Nucleic Acids Res. 14:4683-4690.

Wei, Z. M., and Beer, S. V. 1993. HrpI of *Erwinia amylovora* functions in secretion of harpin and is a member of a new protein family. J. Bacteriol. 175:7958-7967.

Whalen, M. C., Wang, J. F., Carland, F. M., Heiskel, M. E., Dahlbeck, D., Minsavage, G. V., Jones, J. B., Scott, J. W., Stall, R. E., and Staskawicz, B. J. 1993. Avirulence gene *avrRxv* from *Xanthomonas campestris* pv. *vesicatoria* specifies resistance on tomato line Hawaii 7998. Mol. Plant-Microbe Interact. 6:616-627.

Willis, D. K., Rich, J. J., and Hrabak, E. M. 1991. *hrp* Genes of phytopathogenic bacteria. Mol. Plant-Microbe Interact. 4:132-138.

Woestyn, S., Allaoui A., Wattiau, P., and Cornelis, G. R. 1994. YscN, the putative energizer of the *Yersinia* yop secretion machinery. J. Bacteriol. 176:1561-1569.

Wu, H. C., and Tokunaga, M. 1986. Biogenesis of lipoproteins in bacteria. Curr. Top. Microbiol. Immunol. 125:127-157.

Xiao, Y., and Hutcheson, S. W. 1994. A single promoter sequence recognized by a newly identified alternate sigma factor directs expression of pathogenicity and host range determinants in *Pseudomonas syringae*. J. Bacteriol. 176:3089-3091.

Xiao, Y., Heu, S., Yi, J., Lu, Y., and Hutcheson, S. W. 1994. Identification of a putative alternate sigma factor and characterization of a multicomponent regulatory cascade controlling the expression of *Pseudomonas syringae* pv. syringae Pss61 *hrp* and *hrmA* genes. J. Bacteriol. 176:1025-1036.

Zhuang, W. Y., and Shapiro, L. 1995. Caulobacter FlQ and FliR membrane proteins, required for flagellar biogenesis and cell division, belong to a family of virulence factor export proteins. J. Bacteriol. 177:343-356.