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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

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The avirulence gene *avrBs3* from *Xanthomonas campestris* pv. vesicatoria pepper race 1 is responsible for the induction of a race-specific hypersensitive reaction in resistant pepper cultivars. A DNA region of 3.7 kb, containing several open reading frames and an internal repetitive region, was shown previously to be necessary for avirulence activity (U. Bonas, R. E. Stall, and B. Staskawicz, Mol. Gen. Genet. 218:127–136, 1989). The promoter of *avrBs3* was identified by using gene fusions to β-glucuronidase. Also, we mapped the transcription start site and showed that the *avrBs3* gene is expressed constitutively in cells grown in minimal or complex medium and in planta. Polyclonal antibodies raised against a fusion protein produced in *Escherichia coli* allowed the identification of a 122-kDa protein in *X. campestris* pv. vesicatoria cells expressing the *avrBs3* gene. The antibody is specific for AvrBs3 in *X. campestris* pv. vesicatoria cells but also recognizes homologous proteins in other pathovars of *X. campestris*. We found that AvrBs3 is localized intracellularly in *X. campestris* pv. vesicatoria and is mainly in the soluble fraction. The effect of mutations in the *hrp* gene cluster on the function of AvrBs3 was examined. Expression of AvrBs3 in *X. campestris* pv. vesicatoria grown in minimal or complex medium is independent of the *hrp* gene cluster that determines pathogenicity and hypersensitivity to *X. campestris* pv. vesicatoria. In the plant, however, the *hrp* genes are required for elicitation of a race-specific resistance response.

Resistance of pepper plants to infection by *Xanthomonas campestris* pv. vesicatoria, a gram-negative bacterium which is the causal agent of bacterial spot disease of pepper and tomato, is determined by gene-for-gene relationships. Genes for avirulence in particular races of *X. campestris* pv. vesicatoria are matched by genes for resistance in pepper (27). If the host and pathogen lack dominant alleles for resistance or avirulence, tissues are susceptible to bacterial colonization and *X. campestris* pv. vesicatoria multiplies to a high density in the intercellular spaces of leaves, causing water-soaked lesions that later become necrotic (compatible interaction). If matching genes for avirulence and resistance are present, challenged plant cells respond by a hypersensitive reaction (HR; incompatible interaction). The HR is a local defense reaction accompanied by rapid necrosis of the infected tissue, thus preventing multiplication of the bacteria in the infected region (21). Knowledge of the genetics of plant resistance against bacterial spot disease in pepper is confined to classic genetic analyses. In contrast, several avirulence genes have already been isolated from *X. campestris* pv. vesicatoria (3, 15, 27, 30, 39, 41). Similar gene-for-gene relationships have been described in other plant-bacterium interactions, notably between *X. campestris* pv. phaseoli and bean (41), *X. campestris* pv. oryzae and rice (18), *Pseudomonas syringae* pv. pisi and pea (40), and *P. syringae* pv. glycinea and soybean (16). Characterization of the regulation and function of cloned avirulence genes should ultimately elucidate the processes of cell signalling and recognition which underlie the expression of race-specific resistance.

In addition to genes for cultivar- and race-specific avirulence, phytopathogenic bacteria also contain genes associated with the basic ability to grow and cause disease in infected plant tissues. A cluster of genes, designated *hrp* genes, has been found both to determine the ability to elicit the HR in resistant homologous hosts and in heterologous nonhost plants and to control the ability to cause disease. Several *hrp* genes have been identified in *X. campestris* pv. vesicatoria (2, 34) and in pathovars of *P. syringae* pv. phaseolicola (23) and pv. glycinea (12). Investigation of the regulation of the avirulence gene *avrB* from *P. syringae* pv. glycinea has revealed a link between *hrp* genes in the soybean pathogen and the expression of avirulence. It appears that *avrB* is induced in both the plant and minimal medium and that induction is regulated by a locus homologous to the *hrpSR* locus from *P. syringae* pv. phaseolicola (9, 12).

We describe the expression of the *avrBs3* gene in *X. campestris* pv. vesicatoria and present evidence that the *avrBs3* gene encodes a 122-kDa protein. The *avrBs3* gene is located on a self-transmissible plasmid in *X. campestris* pv. vesicatoria race 1 strains, and a region of approximately 3.7 kb was found to be necessary for *avrBs3* activity, i.e., for induction of an HR on pepper cultivar ECW-30R (3). The internal part of the *avrBs3* gene displays an interesting feature: it consists of a tandemly repeated, nearly identical motif of 102 bp that is present 17.5 times (3). The effect of *hrp* genes was studied by analyzing the expression of the protein in different *hrp* mutant backgrounds both in vitro and in resistant pepper plants. Furthermore, we tested for homologous proteins in other pathovars of *X. campestris* by using a specific antibody directed against the AvrBs3 protein.

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TABLE 1. Bacterial strains and plasmids

Designation	Relevant characteristics	Source or reference
X. campestris pv.		
alfalfae KS		D. L. Stuteville[a]
campestris 33913		ATCC[b]
glycines 202		R. E. Stall[c]
malvacearum G-34		M. Essenberg[d]
oryzae PXO86		IRRI[e]
phaseoli 85-6		R. E. Stall
X. campestris pv. vesicatoria		
71-21	Pepper race 1; HR on ECW-30R; carries *avrBs3*	3, 27
82-8	Pepper race 1; HR on ECW-30R; carries *avrBs3*	3, 27
85-10	Tomato and pepper race 2; HR on ECW-10R; carries *avrBs1*	3
E. coli		
DH5α	F⁻ *recA* ϕ80*dlacZ* Δ*M15*	Bethesda Research Laboratories
PK0298	*pro trp his lac* λ⁺ del298 (*manA-uidA*); *uidA* = *gusA*	P. Kuempel[f]
Plasmids		
pLAFR3	Tc^r *rlx*$^+$ RK2 replicon	39
pUR278	β-Galactosidase expression vector; Ap^r	32
pRK2013	Km^r TraRK2$^+$ Mob$^+$ ColE1 replicon	6
pL6GUSC	pLAFR6 derivative containing promoterless β-glucuronidase gene; Tc^r	This paper
pL3XV1-6	*avrBs3* clone in pLAFR3	3
pUXV1006	*avrBs3* subclone in pUC118	3

[a] Kansas State University, Manhattan.
[b] ATCC, American Type Culture Collection, Rockville, Md.
[c] University of Florida, Gainesville.
[d] Oklahoma State University, Stillwater.
[e] IRRI, International Rice Research Institute, Manila, Philippines.
[f] University of Colorado, Boulder.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used are listed in Table 1. Strains of *Escherichia coli* were cultivated in Luria-Bertani (LB) medium (26). *X. campestris* strains were grown in either nutrient or NYG (4) broth. For culture on solid medium, 1.5% agar was added. The minimal medium used was M9 (26). Antibiotics were added to the media at the following final concentrations: ampicillin (Ap), 100 μg/ml; kanamycin (Km), 50 μg/ml; tetracycline (Tc), 10 μg/ml; rifampin (Rif), 100 μg/ml.

Plasmids were introduced into *E. coli* by transformation and into *X. campestris* by conjugation, using pRK2013 as a helper plasmid in triparental matings (5, 6).

Plant material and plant inoculations. Descriptions of the nearly isogenic pepper cultivars ECW, ECW-10R, and ECW-30R have been given by Minsavage et al. (27). Cultivars ECW-10R and ECW-30R carry the dominant resistance genes *Bs1* and *Bs3*, respectively. The leaves of plants were inoculated with bacterial suspensions as described previously (38, 39) by infiltrating the bacteria into the intercellular spaces with a plastic syringe or by vacuum infiltration. For scoring of phenotypic reactions, fully expanded leaves were inoculated. The concentration of the inoculum was approximately 10^8 CFU/ml in 1 mM $MgCl_2$ unless otherwise stated. Reactions were scored over a period of several days.

Intercellular washing fluids were recovered from leaves of pepper plants as described by Klement (20), using 1 mM $MgCl_2$ to resuspend the cells.

Molecular genetic techniques. Standard molecular techniques were used (1, 25) unless otherwise stated.

Construction of promoter fusions and β-glucuronidase assays. Different fragments from the original clone pUXV1006 containing *avrBs3* (3) and deletion clones thereof were fused to the promoterless β-glucuronidase (*gusA*) gene in pL6GUSC. This vector is a derivative of pL6GUSB containing a promoterless *gus* gene flanked by transcriptional terminators and is based on the broad-host-range vector pLAFR1 (3a, 7). Replacement of the *Pst*I-*Eco*RI fragment by the *Pst*I-*Eco*RI fragment isolated from pRAJ255 (13) gave rise to pL6GUSC with unique *Hin*dIII and *Pst*I sites upstream of the β-glucuronidase gene. Fragments containing putative promoter sequences were cloned into the *Pst*I site of pL6GUSC with the *Pst*I sites of the fragments originating from the multicloning site of the pUC vector and the coding region of open reading frame 1 (ORF1; position 704 in the sequence in reference 3).

To generate in situ fusions of *avrBs3* to the β-glucuronidase gene, we introduced different Tn*3-gus* insertions, obtained in the insert of pL3XV1-6 previously (3), into the genome of *X. campestris* pv. vesicatoria race 1 strains 71-21 and 82-8 by marker gene replacement, as described before (2). The mutants were analyzed in Southern hybridization experiments and by inoculation into pepper leaves.

For β-glucuronidase assays, bacteria grown in NYG broth were harvested by centrifugation and resuspended in assay buffer. For β-glucuronidase assays of in planta-grown bacteria, pepper leaves were inoculated with 5×10^7 CFU/ml in 1 mM $MgCl_2$. At intervals, leaf disks (0.6 cm in diameter) were each macerated in 100 μl of 1 mM $MgCl_2$; a 50-μl sample of the extract was taken for the assay. The number (CFU) of bacteria per assay was calculated by plating

appropriate dilutions of the leaf disk extracts on the corresponding medium. β-Glucuronidase activity was determined in fluorometric assays, using 4-methyl umbelliferyl glucuronide as substrate as described by Jefferson et al. (14). Enzyme activity (1 unit = nanomoles per minute) was measured at different points after the start of incubation and of triplicate samples per time point. Controls included the corresponding parental *X. campestris* pv. vesicatoria strains carrying pL6GUSC or pL3GUS, a pLAFR3 (37) derivative with the *lacZ* promoter driving expression of the β-glucuronidase gene (3a), and, for in planta assays, leaf disks of uninfected pepper tissue.

RNA isolation. Bacterial cells were harvested by centrifugation, resuspended in water containing 1% diethylpyrocarbonate, and lysed at 100°C for 30 s by adding 4 volumes of boiling lysis buffer (50 mM Tris HCl [pH 9], 50 mM EDTA, 0.3 M sodium acetate, 0.6% sodium dodecyl sulfate [SDS]). After two extractions each with phenol (65°C) and chloroform, the nucleic acids were precipitated with 1 volume of isopropanol and resuspended in water. The RNA was precipitated by incubating for 1 h in 2 M lithium acetate (twice). Finally, the RNA was ethanol precipitated and resuspended in water.

Primer extension. The *Bam*HI-*Pst*I restriction fragment, radioactively labelled by filling in the 3′ end using [α-^{32}P]dGTP, was denatured and mixed with 20 to 50 μg of total RNA. This annealing mixture was precipitated, resuspended in 50 μl of buffer {40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] [pH 6.4], 1 mM EDTA, 0.4 M NaCl, 50% formamide}, and incubated overnight at 50°C. The primer extension reaction and analysis of the products were performed as described previously (25).

Preparation of antisera. Translational fusions between the *E. coli* β-galactosidase gene and portions of the *avrBs3* gene were constructed in the pUR series of vectors containing a multicloning site at the 3′ end of the β-galactosidase gene. The pUR plasmids express the 116-kDa β-galactosidase protein under the control of the *lacZ* promoter in the presence of IPTG (isopropyl-β-D-thiogalactopyranoside) (31). A 2.9-kb deletion fragment, starting at the N-terminal *Bam*HI site of the *avrBs3* gene and spanning the major part of ORF1 except for the C-terminal region, was cloned into the expression vector pUR278 (plasmid pUR9). Induced *E. coli* cells carrying pUR9 strongly expressed a protein of 200 kDa which was isolated for immunization of rabbits. The fusion site of the construct was checked by DNA sequence analysis. Fusion proteins were expressed in *E. coli*, extracted by sonification, and purified from SDS-polyacrylamide gels for immunization of New Zealand White rabbits.

Since expression of fusion proteins containing sequences corresponding to ORF2 were unsuccessful, a synthetic peptide was used for immunization. The sequence of the peptide, ATVSKASRARAPDHC, corresponded to a C-terminal region in the protein sequence deduced from ORF2 and was predicted to be highly antigenic.

To raise polyclonal antiserum, the first injection contained ca. 250 μg of protein in 50% Freund complete adjuvant; booster injections contained ca. 125 μg of protein in 50% incomplete adjuvant (Sigma). The crude antifusion antisera were affinity purified to the fusion protein immobilized on nitrocellulose (36). Antibodies to the β-galactosidase portion of the fusion protein were removed by adsorption to an *E. coli* extract containing β-galactosidase. The remaining antibodies were used in Western blot (immunoblot) analysis at a dilution of up to 1:15,000 relative to the original antiserum.

Protein analysis, immunoblotting, and protein fractionation. *E. coli* cells carrying pUR plasmids were grown in LB broth with ampicillin, induced with 1 mM IPTG at an A_{600} of 0.2, and harvested 6 h after induction. Preparative isolation of bacterial fusion proteins was essentially as described previously (29). The extraction buffer used was 50 mM Tris HCl (pH 7.5)–0.2 M NaCl–5 mM EDTA–2 mM 2-mercaptoethanol–100 μg of phenylmethylsulfonyl fluoride per ml. Insoluble proteins were separated from *E. coli* extracts by centrifugation at 237,000 × *g* (2 h; 4°C), washed, and solubilized in Laemmli buffer (22).

Isolation of proteins from *X. campestris* cells for fractionation studies was performed at 4°C as follows. Cells grown in nutrient broth were harvested at an A_{600} of 0.7 to 0.9 by centrifugation, washed with 50 mM Tris HCl (pH 7.5), and resuspended in cold extraction buffer (ca. 3 ml/g of cells). After addition of RNase A and DNase I each to a final concentration of 10 μg/ml and proteinase inhibitor mix (Chymostatin, Antipain, Aprotenin, Pepstatin A, Leupeptin; from Sigma) to 1 μg/ml, cells were broken by three passages through a prechilled French pressure cell (15,000 lbs/in^2). Unbroken cells were removed by centrifugation. The cell extracts were separated into soluble and insoluble material by centrifugation at 237,000 × *g* for 4 h at 4°C. For the isolation of total membranes, total extract was layered on top of a 60–25% sucrose cushion. The other part of the extract was diluted with extraction buffer to analyze the soluble proteins in the supernatant. A total membrane fraction was extracted from the sucrose gradient by puncturing the tube with a needle.

For analytical protein analysis, *E. coli* or *X. campestris* cells were harvested by a centrifugation step and resuspended in Laemmli buffer (22). After samples were boiled for 90 s and centrifuged for 5 min, they were separated on 6 or 7% discontinuous SDS-polyacrylamide gels, using bisacrylamide or *N*,*N*′-diallyltartardiamide (1.2% in 29.2% acrylamide) as cross-linkers. The gels were stained with Coomassie blue R-250 or transferred to nitrocellulose by electroblotting in a buffer containing 0.192 M glycine, 0.025 M Tris base (pH 8.3), 20% methanol, and 0.1% SDS. Western blots were reacted overnight with primary antibody. For detection of bound antibody, we used alkaline phosphatase-conjugated goat antibodies to rabbit immunoglobulin G (Promega Biotec).

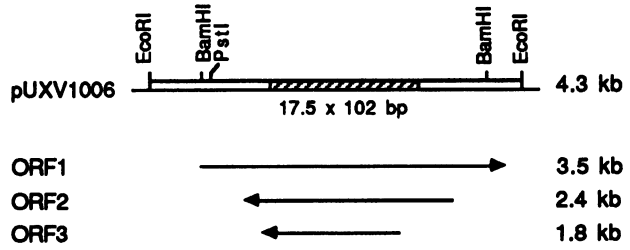

FIG. 1. Structure of the DNA region containing *avrBs3*. Clone pUXV1006 carries a 4.3-kb *Eco*RI insert (open box) which was sequenced previously (3). The hatched box represents the internal region consisting of a tandemly repeated, nearly identical, 102-bp sequence which is present 17.5 times. The longest ORFs and their orientation and size are indicated by the arrows.

RESULTS

Identification of the *avrBs3* promoter. The nucleotide sequence of a 4.3-kb fragment containing the *avrBs3* gene

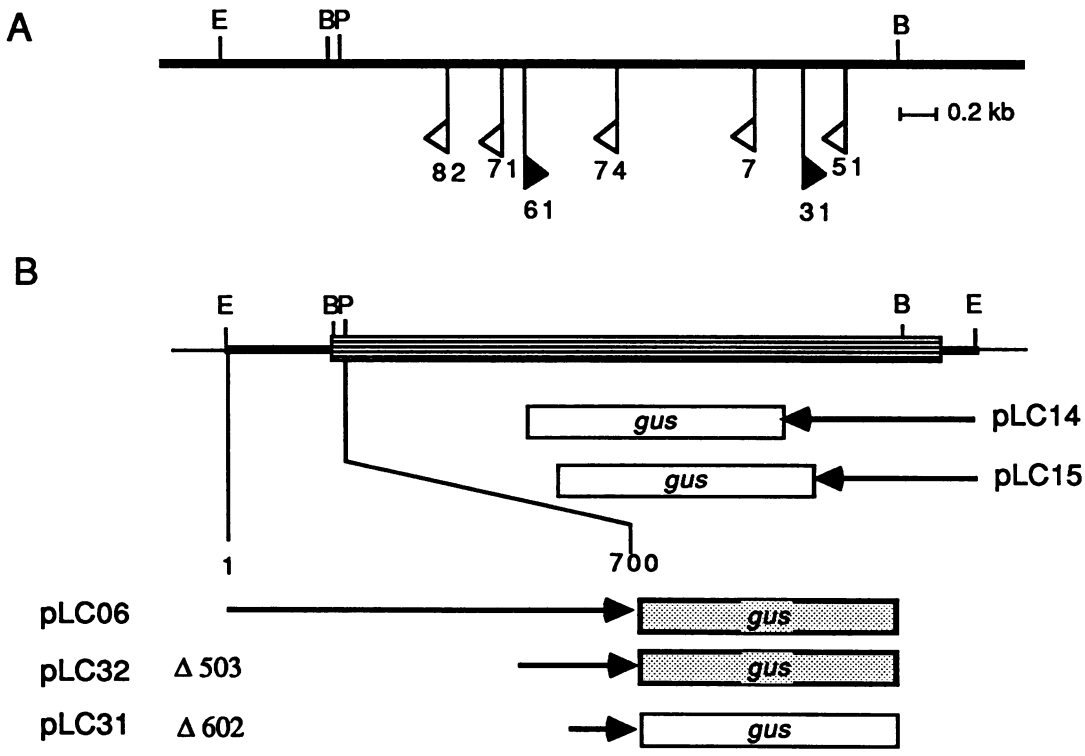

FIG. 2. Fusion of *avrBs3* sequences to the β-glucuronidase gene (*gusA*). (A) Position, orientation, and activity of the *gus* gene in single Tn*3-gus* insertions in *avrBs3* of *X. campestris* pv. vesicatoria race 1 strains 71-21 and 82-8, obtained by marker gene replacement in the genome. Insertions marked with an open triangle did not reveal β-glucuronidase activity, while insertions 31 and 61 showed β-glucuronidase activity. (B) The uppermost line represents the insert of pUXV1006. ORF1 (positions 621 to 4112) is represented by the striped box. pLC06, pLC32, and pLC31 are clones in pL6GUSC, containing fragments from the upstream region of ORF1 (indicated by arrows), fused to the promoterless *gusA* gene. The right border of these fragments is the *Pst*I site at position 700. Clones pLC14 and pLC15 contain fragments from the upstream region of ORF2 and ORF3. Dark boxes containing *gus* represent β-glucuronidase-positive constructs; light boxes represent β-glucuronidase-negative constructs. E, *Eco*RI; B, *Bam*HI; P, *Pst*I site.

revealed the presence of several long ORFs (Fig. 1) (3). The positions of these ORFs were unaffected by a minor sequence mistake in the sequence published in reference 3 (corrected sequence in EMBL accession no. X16130). To determine whether the *avrBs3* gene is expressed in the direction of ORF1 or from the opposite DNA strand which contains ORF2 and ORF3, single-stranded DNA fragments were hybridized with total RNA from *X. campestris* pv. vesicatoria race 1 cells. Only fragments complementary to the ORF1-encoding DNA strand hybridized, indicating expression in the direction of ORF1 (data not presented). We then generated genomic marker gene exchange mutants in *X. campestris* pv. vesicatoria race 1 strains 71-21 and 82-8, using different Tn*3-gus* insertions in the *avrBs3* gene. Function of *avrBs3* was lost in all mutants. Tn*3-gus* carries a promoterless β-glucuronidase gene (*gusA*) (3). The orientation and the level of β-glucuronidase expression in Tn*3-gus* insertion mutants 82, 71, 61, 74, 7, 31, and 51 was determined after growth of the bacteria in medium or in pepper leaves (Fig. 2A). In no case was β-glucuronidase activity measured when the *gusA* gene was oriented in the direction of ORF2 or ORF3. Only insertion mutants 31 and 61, oriented in the direction of ORF1, clearly showed β-glucuronidase activity (see below).

DNA fragments containing putative promoter regions were cloned in front of the *gusA* gene in pL6GUSC. For the isolation of the putative ORF1 promoter, *Pst*I fragments from the full-length *avrBs3* clone pUXV1006 and the deletion derivatives pUXV1632 and pUXV1631 were cloned into pL6GUSC to give rise to pLC06, pLC32, and pLC31 with insert sizes of 710, 220, and 110 bp, respectively (Fig. 2B). For ORF2 and ORF3 the inserts of smaller deletion clones derived from pUXV1006 were used to give rise to pLC14 (1,140-bp insert) and pLC15 (970-bp insert; Fig. 2B).

The constructs were introduced into *X. campestris* pv. vesicatoria race 1 strain 82-8 and race 2 strain 85-10 by triparental mating. The β-glucuronidase activities of the transconjugants were determined at various times after inoculation of the cells in NYG broth or in planta. To see whether the plant genotype (susceptible or resistant) influenced *avrBs3* promoter activity, transconjugants of strains 82-8 and 85-10 were inoculated into pepper cultivars ECW (susceptible to both) and ECW-30R (resistant to 82-8). Only DNA fragments originating from the 5′ DNA region of ORF1 showed detectable promoter activity (Fig. 2B). The level of β-glucuronidase activity was constant under the different conditions tested. Significantly, there was no indication of induction of *avrBs3* in the plant. The values for the constructs pLC06 and pLC32 were 0.6×10^{-8} to 1.8×10^{-8} and 0.2×10^{-8} to 1.2×10^{-8} U CFU^{-1}, respectively. Higher activities were obtained with cells in the stationary growth phase. The values for these constructs were higher than the activities found with the marker exchange Tn*3-gus* mutants 31 and 61 (0.3×10^{-8} and 0.08×10^{-8} U CFU^{-1}, respectively). This is most likely due to the difference in copy number of the gene fusions tested. When the 5′ region was

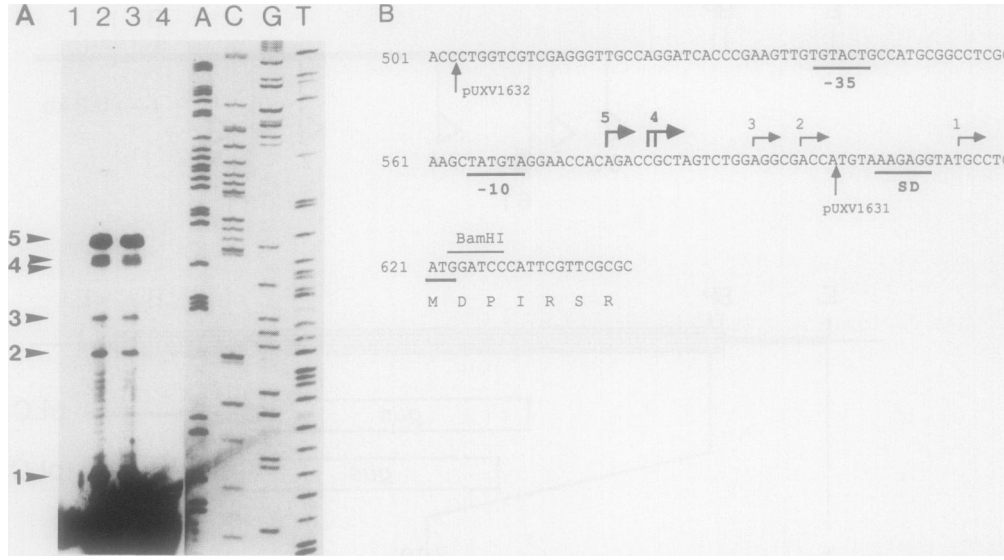

FIG. 3. (A) Autoradiograph of a primer extension experiment to map the transcription start site of ORF1. Lane 1, 77-bp *Bam*HI-*Pst*I primer. Lanes 2, 3, and 4, extension products using total RNA from *X. campestris* pv. vesicatoria 71-21, 82-8, and 85-10, respectively. The bacteria were grown in NYG broth. Lanes A, C, G, and T, dideoxy sequence reactions of M13mp8 as a size marker. Samples were separated on a 6% acrylamide gel. The numbers to the left of the arrows mark the strongest extension signals. (B) Sequence of ORF1 promoter region. The sequence of *avrBs3* is shown, starting at position 501 (see reference 3). Positions of putative start sites of transcription are indicated by horizontal arrows; numbers 1 through 5 refer to the bands visible in panel A. Vertical arrows mark the beginning of deletion clones pUXV1632 and pUXV1631 used for promoter studies (Fig. 2B). Sequence homologies to −35 and −10 promoter elements and Shine-Dalgarno sequences (SD) are underlined. The ATG codon at position 621 is the putative start of translation.

deleted further, as in pLC31, promoter activity was lost. The sequences upstream of ORF2 and ORF3, present in constructs pLC14 and pLC15, had little (0.8×10^{-10} U CFU^{-1}) or no detectable promoter activity in race 1 or race 2 transconjugants under all conditions tested. To test whether the *avrBs3* promoter is active in *E. coli*, the constructs pLC06 and pLC32 were also tested in the *E. coli gusA* strain PK0298. No promoter activity was detected for pLC32, while transformants carrying pLC06 showed a β-glucuronidase activity of 2.5×10^{-10} U CFU^{-1}.

Thus, the region upstream of ORF1 contains a promoter that is constitutively active in *X. campestris* pv. vesicatoria. A sequence starting 45 bp upstream of the first ATG in ORF1 and 117 bp upstream of the second ATG in ORF1 and ending 85 bp downstream of the second ATG was sufficient for promoter activity. This is in good agreement with the previous finding that the deletion clone pUXV1631 was found to be inactive (no HR in ECW-30R) whereas clone pXV1632 still had avirulence activity (3). Furthermore, the promoter activity was not dependent on the type of race or plant cultivar used.

Mapping of the transcription start site. The promoter studies described above strongly suggested transcription in the direction of ORF1. To map the transcription start site, total RNA was extracted from cells of race 1 strains 71-21 and 82-8 and, as a negative control, race 2 strain 85-10, grown in NYG broth and in pepper leaves, respectively. For primer extension experiments, the 77-bp *Bam*HI-*Pst*I restriction fragment spanning the region from nucleotide positions 624 to 700 was used as a primer to synthesize a cDNA strand corresponding to the RNA. As shown in Fig. 3A, the RNAs from strains 71-21 and 82-8 gave rise to identical reaction products (lanes 2 and 3). The same pattern was obtained repeatedly with different RNA preparations from cells grown in either NYG broth or pepper ECW. The RNA from *X. campestris* pv. vesicatoria 85-10 (lane 4) did not result in any primer extension products. In Fig. 3B the sequence of the region of *avrBs3* is shown in which the positions corresponding to the putative transcription start sites and promoter control elements are indicated. The position of the strongest primer extension signal (Fig. 3A and B, position 5) coincides well relative to the position of sequences resembling prokaryotic −10 and −35 consensus promoter elements. Interestingly, the −10 sequence element in the promoter of *avrBs3* was identical to the one found in the putative promoter region upstream of the avirulence gene *avrBs1* from *X. campestris* pv. vesicatoria race 2 (30). We therefore conclude that transcription of the *avrBs3* gene starts around position 580. Downstream, a sequence with homology to a putative ribosome-binding site (Shine-Dalgarno [SD]; 35) is present, indicating that translation may start at the ATG indicated at position 621 (Fig. 3B).

Detection of the *avrBs3* protein. To visualize the protein product of the *avrBs3* gene, polyclonal antisera were generated by immunizing rabbits with a β-galactosidase–AvrBs3 fusion protein containing the major part of ORF1 except for the C-terminal region. The antiserum was affinity purified, and Western blot analysis (Fig. 4) reveals that the antibody specifically recognized a protein with a relative mobility of approximately 125 to 130 kDa in cell extracts of *X. campestris* pv. vesicatoria race 1 cells. These data are in agreement with the 122-kDa protein predicted for the protein encoded by ORF1. Protein extracts from *X. campestris* pv. vesicatoria race 2 did not react with the antibody. However, a race 2 transconjugant harboring the *avrBs3* gene in plasmid pL3XV1-6 and having avirulence activity on ECW-30R expressed the 122-kDa protein (Fig. 4, lane 3). No signals were obtained in Western blots of proteins recovered from culture supernatants of *X. campestris* pv. vesicatoria cells expressing AvrBs3 or from intercellular washing fluids of

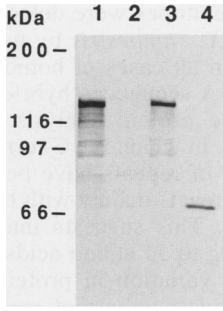

FIG. 4. Western blot analysis of AvrBs3 in *X. campestris* pv. vesicatoria. Total protein extracts of cells (grown in NYG broth) from *X. campestris* pv. vesicatoria race 1 strain 71-21 (lane 1), race 2 strain 85-10 (lane 2), 85-10 transconjugant harboring *avrBs3* on pL3XV1-6 (lane 3), and 71-21 marker exchange mutant Tn*3-gus* 74 (lane 4) were separated on a 7% polyacrylamide gel and electrophoretically transferred to nitrocellulose. The blot was reacted with affinity-purified antiserum directed against AvrBs3 (1:10,000 dilution). Bound antibody was visualized by reaction with alkaline phosphatase-conjugated secondary antibody.

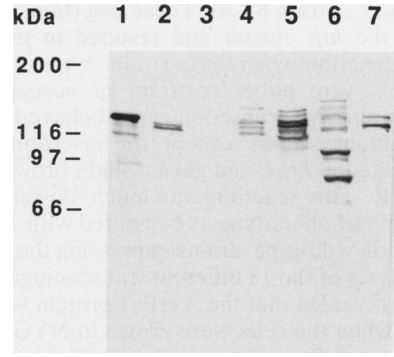

FIG. 5. Western blot analysis of protein extracts from different pathovars of *X. campestris*. Total protein extracts of cells (grown in NYG broth) from *X. campestris* pv. vesicatoria race 1 strain 71-21 (lane 1) were compared with extracts from pathovars of *X. campestris* pv. alfalfae (lane 2), campestris (lane 3), glycines (lane 4), malvacearum (lane 5), oryzae (lane 6), and phaseoli (lane 7). The experiment was performed as described in the legend to Fig. 4.

pepper leaves after infection with *X. campestris* pv. vesicatoria race 1.

To confirm that the antibody was directed against the 122-kDa protein, a number of mutants deleted in the C-terminal portion of the *avrBs3*-gene or marker exchange Tn*3-gus* insertions, all resulting in loss of activity, were tested. One example is shown in Fig. 4, lane 4. Tn*3-gus* insertion mutant 74 in *X. campestris* pv. vesicatoria race 1 expressed a protein of about 60 kDa, which is in good agreement with the size predicted on the basis of the site of gene disruption (Fig. 2A). Preimmune sera did not react with any of these proteins.

Although the promoter studies did not reveal an active promoter for the ORF2 upstream region under the conditions used, we wanted to test whether the corresponding peptide, Pep2, could be identified in *X. campestris* pv. vesicatoria race 1 cells. Attempts to express Pep2 or part of the protein in *E. coli* by using different expression vectors were unsuccessful, which may have been because of the unusual codon usage of ORF2 (3). Therefore, a polyclonal antibody was raised against a synthetic peptide corresponding to a region in the C terminus of Pep2. The antibody specifically recognized the synthetic peptide in dilutions of up to 1:5,000; however, it did not react with any particular protein in cell extracts from *X. campestris* pv. vesicatoria race 1 grown in NYG broth or in planta. Taken together with the promoter studies, these results suggest that ORF2 is not expressed. We conclude, therefore, that the protein expressed from the *avrBs3* gene, AvrBs3, and responsible for the avirulence activity on pepper cultivar ECW-30R is derived from ORF1.

The availability of a specific antibody directed against AvrBs3 allowed us to ascertain the subcellular localization of the protein. For biochemical fractionation, cells of *X. campestris* pv. vesicatoria race 1 were disrupted by a French pressure cell, and the extracts were separated into soluble and insoluble proteins. AvrBs3 was mainly present in the soluble fraction of the cell, although on average 20 to 30% of the total amount of the AvrBs3 signal was estimated to be associated with the membrane fraction (data not presented). The same result was obtained when cell extracts were prepared in the presence of 0.2 M NaCl, which reduces hydrophobic interactions of proteins. The amount as well as the localization of AvrBs3 were unaltered when the distribution of the proteins were tested after growth of *X. campestris* pv. vesicatoria in the plant.

Homologous proteins in other pathovars of *X. campestris*. DNA hybridization experiments performed previously revealed the presence of DNA sequences homologous to the 3.3-kb *Bam*HI fragment, spanning the internal part of the *avrBs3* gene, including the repetitive region, in a number of other pathovars of *X. campestris*, namely, alfalfae, campestris, carotae, glycines, malvacearum, and phaseoli (3). This finding prompted a search for homologous protein sequences that could be detected with the AvrBs3-specific antibody. Protein extracts were prepared from cells of different pathovars of *X. campestris* after growth in NYG broth. As shown by Western blotting (Fig. 5), the AvrBs3 antibody recognized epitopes not only in protein extracts from *X. campestris* pv. vesicatoria race 1 strain 71-21 (Fig. 5, lane 1) but also from *X. campestris* pv. alfalfae (lane 2), glycines (lane 4), malvacearum (lane 5), oryzae (lane 6), and phaseoli (lane 7). No signals were evident in protein extracts from pv. carotae (not shown) and campestris (Fig. 5, lane 3), although the DNA of pv. campestris contained strongly hybridizing sequences to the internal portion of the *avrBs3* gene (see reference 3). Since the cells of the different strains used for these studies were grown in vitro, the proteins detected by the antibody seem to be constitutively expressed, as is AvrBs3. Most of the proteins reacting with the AvrBs3 antibody were in the same size range as the AvrBs3 protein. *X. campestris* pv. glycines, malvacearum, and oryzae, however, also expressed larger cross-reacting proteins. The strongest signals in the extracts from *X. campestris* pv. oryzae corresponded to proteins of ca. 100 and 80 kDa.

Function of *avrBs3* depends on *hrp* genes. We recently isolated a *hrp* gene cluster from *X. campestris* pv. vesicatoria containing at least six different *hrp* loci (*hrpA* to *hrpF*) (2). These genes are necessary for pathogenicity on susceptible hosts and for induction of the HR during incompatible interactions on host or nonhost plants. The expression studies of the *avrBs3* gene showed that it is expressed constitutively. To test whether all *hrp* loci are necessary for *avrBs3* function, i.e., induction of the HR in pepper cultivar ECW-30R, plasmid pL3XV1-6 containing the *avrBs3* gene was introduced into 18 different Tn*3-gus* insertion mutant

strains of race 2 strain 85-10. These insertions were located throughout the *hrp* cluster and resulted in the loss of all phenotypic reaction when these strains were inoculated into plants which were either resistant or susceptible to the wild-type strain. All transconjugants behaved like the *hrp* insertion mutant strains except for insertion mutant 75, which is located in *hrpE* and gave a slight browning reaction on ECW-30R. This reaction was much slower and did not reach the full HR phenotype as compared with *X. campestris* pv. vesicatoria wild-type strains expressing the *avrBs3* gene. Protein analysis of the 18 different transconjugants by Western blotting revealed that the AvrBs3 protein was expressed in all cases when the cells were grown in NYG broth. Since the *hrp* mutants fail to grow in the plant (2), expression of the *avrBs3* gene under these conditions could not be determined. When *hrp* mutant strains carrying *avrBs3* and the wild-type race 2 strain 85-10 were coinoculated into ECW-30R, no complementation for HR induction was observed.

DISCUSSION

X. campestris pv. vesicatoria strains containing the *avrBs3* gene induce an HR specifically on plants of pepper cultivar ECW-30R, which carries the resistance locus *Bs3* (3, 27). Previously, genetic studies showed that the activity of the gene was confined to a 3.7-kb DNA fragment (3). Here we describe the expression of the *avrBs3* gene. Using precise DNA fusions of putative promoter fragments, and also randomly obtained fusions to the β-glucuronidase gene as a reporter gene, we show that ORF1 is expressed and identify a promoter region in the upstream sequence of this ORF (Fig. 2). The promoter showed constitutive activity under the different growth conditions tested. This finding is in contrast to other avirulence genes from *X. campestris* (30) or *Pseudomonas* (12) strains that are only expressed after the interaction of the bacteria with the plant or, as shown for *avrB* (12), by growth of the bacteria in minimal media. Since deletions affecting ORF1 resulted in loss of avirulence activity and no expression of ORF2 or ORF3 was detected, we believe that the avirulence protein, AvrBs3, is expressed from ORF1. Primer extension experiments predicted a region around position 580 in the sequence of *avrBs3* as the start of transcription of the RNA corresponding to ORF1 (Fig. 3A and B). These results supported conclusions reached from the promoter studies. Sequence comparisons to putative promoter regions of *avrBs1* from *X. campestris* pv. vesicatoria (30), an endoglucanase gene from *X. campestris* pv. campestris (8), and other prokaryotic genes suggested putative −10 and −35 promoter elements around positions 569 and 544, respectively. Because of a lack of information on transcription initiation of other genes from *Xanthomonas* spp. sequenced so far, no bona fide *Xanthomonas* promoter has yet been defined. The sequence at positions 607 to 612 resembles the Shine-Dalgarno consensus sequence from *E. coli* (35) and is located 10 bp upstream of the second ATG codon in ORF1 so that the translation of the AvrBs3 protein presumably starts at position 621 and ends at position 4112, giving rise to a protein of 1,164 amino acids.

Western blots of total extracts of *X. campestris* pv. vesicatoria cells with *avrBs3* activity showed that a 125-kDa protein is expressed and specifically recognized by an affinity-purified polyclonal antibody directed against a major portion of AvrBs3 (Fig. 4). The apparent size of this protein (125 to 130 kDa) is in good agreement with the size predicted (122 kDa) by the DNA sequence of *avrBs3* ORF1. Interestingly, homologous epitopes were detected in proteins from other pathovars of *X. campestris* by using the anti-AvrBs3 antibody (Fig. 5). In all cases of homology, we had previously observed DNA sequences hybridizing to *avrBs3* (3). We know from the protein analysis of various AvrBs3 deletion derivatives, in which the C-terminal part of AvrBs3 and/or the majority of repeats have been deleted, that the antibody used here reacts mainly with the repetitive portion of the protein (1a). This suggests that the 102-bp repeat motif, corresponding to 34 amino acids, is present in other pathovars and that variation in protein size (Fig. 5) may simply reflect differing numbers of repeats. Whether these proteins represent products of alleles of the *avrBs3* gene that are involved in race specificity of these pathovars on their respective host plants remains to be determined. In this context it is intriguing that deletion of parts of the repetitive region of *avrBs3* has been found to confer different patterns of induction of the HR to transconjugants of *X. campestris* pv. vesicatoria harboring the modified genes (10a). All pathovars of *X. campestris* mentioned above induce a nonhost HR on different pepper cultivars, but the response is quite distinct from the HR phenotype induced by the *avrBs3* gene (3). Since *avrBs3* is localized on a self-transmissible plasmid in *X. campestris* pv. vesicatoria race 1 strains (3), it is possible that the gene may have been transferred by conjugation between different pathovars of *X. campestris* and, being expressed in another genetic background, generates a different plant response.

Biochemical fractionation experiments of *X. campestris* pv. vesicatoria race 1 cells showed that AvrBs3 is predominantly a soluble protein and is present intracellularly. This was predicted by the sequence analysis, although the internal part of the protein comprising the repeated units of 34 amino acids is less hydrophilic than the N-terminal and the C-terminal portions of AvrBs3. There is, however, no prediction of a signal peptide sequence or of a membrane-spanning domain in the protein. By contrast, other proteins with repetitive structures, identified in a number of organisms and different in function, have been shown or predicted to be associated with membranes (e.g., see references 10, 11, 19, and 24). Whether the amount of AvrBs3 found in the membrane fraction of *X. campestris* pv. vesicatoria cells is significant and indicates that part of the protein is indeed associated with membranes or whether this is a preparational artifact is not clear. Further analysis of the protein localization in ultrathin sections of *X. campestris* pv. vesicatoria race 1 cells, using gold-labelled secondary antibody, are in progress.

As AvrBs3 seems to be an intracellular protein, there remains the intriguing question: How does the protein function to induce the HR in pepper plants with the matching *Bs3* resistance gene? All other avirulence genes isolated to date from bacteria have similarly been predicted from the sequence to produce soluble proteins; however, no localization studies have been performed with other gene products. No excretion or secretion of the avirulence protein was observed with *X. campestris* pv. vesicatoria, but this has been shown for the product of *avr9*, a race-specific avirulence gene from the pytopathogenic fungus *Cladosporium fulvum* (33).

The only example of the production of a race-specific elicitor of the HR by bacteria is in the interaction of soybean with *P. syringae* pv. glycinea expressing the *avrD* gene (17). This elicitor is a low-molecular-weight compound whose production is regulated by *avrD*; the AvrD protein itself does not elicit the HR. The *avrD* elicitor is produced by the

bacteria in vitro, even in the absence of functional *hrp* genes. However, for in planta production of the HR-inducing elicitor, *hrp* functions are required (17). The need for *hrp* genes, e.g., *hrpSR*, a putative regulatory locus (9, 12, 28), has also been shown for the expression of *avrB* from *P. syringae* pv. glycinea, the function of which is as yet unknown (12). We demonstrated here that, although the *avrBs3* gene is expressed in vitro when introduced into an *hrp* mutant background, its function in planta is dependent on *hrp* genes, except perhaps *hrpE*. These results indicate that the *hrp* genes may provide some basic metabolic functions which indirectly influence the activity of AvrBs3 protein produced when the bacteria are growing in the plant. We cannot rule out that our failure to detect AvrBs3 outside bacterial cells is a matter of the detection limits given by the antibody used. It is conceivable that only a small extracellular amount of the protein is necessary for induction of the HR and/or that these molecules are tightly bound to plant material. One could therefore speculate that Hrp proteins are directly interacting with AvrBs3, e.g., by providing for transport of this protein through the cell wall once the bacteria have entered the plant. This hypothesis would fit well with the finding that the expression of the *hrp* genes from *X. campestris* pv. vesicatoria is plant inducible (33a, 34), and therefore AvrBs3 would not be transported within cells grown in vitro. Clearly, further studies on the functional domains of the AvrBs3 protein will shed more light on its role in the interaction with resistant pepper plants.

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